PRIMAQUINE, SN 13272, A NEW CURATIVE AGENT IN *VIVAX* MALARIA: A PRELIMINARY REPORT[1]

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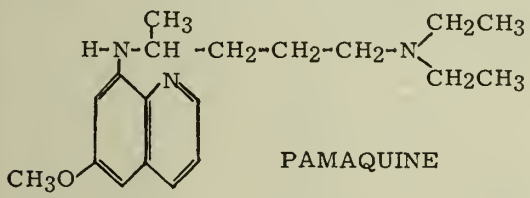
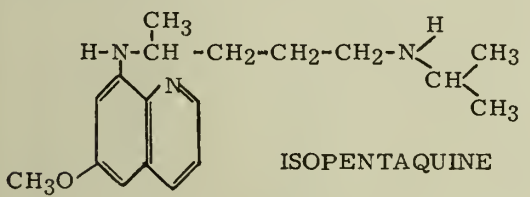
This paper reports the preliminary clinical experience with primaquine, 8-(4-amino-1-methylbutylamino)-6-methoxyquinoline, or SN 13,272, in reducing the relapse rate of the Chesson strain of *vivax* malaria. This compound was first tested in man early in 1948 as part of a comprehensive study of compounds related to pamaquine, using methods previously described by Alving, et al. (1948).

Primaquine differs chemically from pamaquine by having a primary amine substituted for the tertiary terminal amine on the aliphatic side chain in the 8-position of the quinoline nucleus. Pamaquine, isopentaquine and the new compound, primaquine, may be considered as a family of compounds, the members of which differ only in he characteristics of the terminal amino group. Thus:



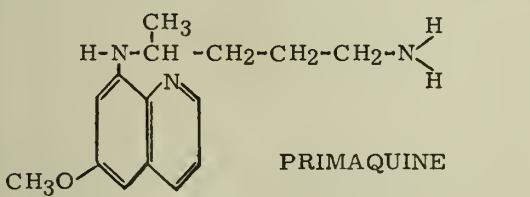
(Tertiary terminal amine)

PAMAQUINE



(Secondary terminal amine)

ISOPENTAQUINE



(Primary terminal amine)

PRIMAQUINE

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PROCEDURES AND METHODS

In the therapeutic trials primaquine was given in the form of the diphosphate salt (56.9 per cent base) and doses have been calculated in terms of base weight.[3]

TABLE 1

Relapse rate after treatment of primary attacks of Chesson strain vivax *malaria (standardized sporozoite infections) with suppressive drugs*

DRUG	RELAPSE RATE
Quinine	18/18
Quinacrine	4/4
Chlorguanide	8/8
Chloroquine	8/8
Total	38/38

TABLE 2

Curative effect of primaquine when administered alone during primary attacks of vivax *malaria (Chesson strain)*

CASES	DAYS FROM END OF Rx TO FIRST RELAPSE		FOLLOW UP	RATIO: SUBJECTS RELAPSED/ SUBJECTS TREATED	SYMPTOMS	MET* HGB	MEAN PLASMA† CONCENTRATION
	F/102+	Para.					
Daily dose of 22.5 mgm.‡							
			days			*per cent*	*gamma/liter*
1	16	15	—		None	9.6	9
2	17	14	—		Abd. +	9.4	10
3	12	13	—	4/5	None	13.9	5
4	16	12	—		None	15.8	4
5	—	—	330		Abd. ++	8.0	4
Daily dose of 45 mgm.‡							
1	—	—	292		None	20.0	43
2	18	16	—		Abd. ++	22.0	75
3	—	—	333	1/5	None	18.1	45
4	—	—	333		Abd. +	16.9	33
5	—	—	284		Abd. +	23.8	30

* Expressed as per cent of total hemoglobin (average for last 5 days of treatment).

† Determined by method of Brodie, Udenfriend and Taggart (1947).

‡ Drug administered in 6 divided doses daily for 2 weeks.

Abd. + = mild, transient abdominal cramps.

Abd. ++ = moderate, repeated abdominal cramps.

participation of Army Medical Officers assigned to the project. The studies would not have been possible except for the valuable cooperation and help given by Warden Joseph E. Ragen of Stateville Penitentiary and other adminitrative officials of the State of Illinois.

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[3] Primaquine was first synthesized by Dr. Robert C. Elderfield, Department of Chemistry, Colum-

The details of the testing program have been reported elsewhere. Briefly the general procedure was as follows: White, healthy inmate volunteers from the general population of the Illinois State Penitentiary (Stateville Branch) who had had no previous experience with malaria were selected. A standard method of inoculation by the bites of ten infected mosquitoes was used because this gives a consistently severe

TABLE 3

Curative effect of primaquine when administered together with 1.64 gms. quinine daily in primary attacks of vivax *malaria (Chesson strain)*

CASES	DAYS FROM END OF Rx TO FIRST RELAPSE		FOLLOW UP	RATIO: SUBJECTS RELAPSED/ SUBJECTS TREATED	SYMPTOMS	MET* HGB	MEAN PLASMA† CONCENTRATION OF PRIMAQUINE
	F/102+	Para.					
				Daily dose of 15 mgm.‡			
			days			*per cent*	*gamma/liter*
1	19	16	—		None	4.8	13
2	66	65	—		Anorexia	15.1	32
3	58	57	—	4/5	None	2.2	14
4	50	47	—		None	4.9	12
5	—	—	451		None	3.3	12
				Daily dose of 30 mgm.‡			
1	—	—	370		Abd. +	8.7	12
2	—	—	369		None	12.1	15
3	—	—	365	0/5	None	9.8	11
4	—	—	365		None	11.0	14
5	—	—	356		None	7.7	13
				Daily dose of 60 mgm.‡			
1	—	—	403		Abd. +++	8.2	27
2	—	—	405	0/4	Abd. +	6.8	58
3	—	—	377		Abd. ++	9.1	42
4	—	—	367		Abd. ++	9.2	21

* Expressed as per cent of total hemoglobin (average for last 5 days of treatment).

† Determined by method of Brodie, Udenfriend and Taggart (1947).

‡ Drug administered in 6 divided doses daily for 2 weeks.

Abd. + = mild, transient abdominal cramps.

Abd. ++ = moderate, repeated abdominal cramps.

Abd. +++ = severe, persistent abdominal cramps.

infection. Drug administration was begun early in the course of the clinical attack in order to reduce acquired immunity to a minimum. During the therapeutic trials the patients were hospitalized. Parasite counts were made daily during the immediate follow-up period and at frequent intervals for periods up to one year. No case with

bia University, New York City. Later supplies of primaquine have been provided by Eli Lilly and Company, Indianapolis, Indiana and by the Abbott Laboratories, North Chicago, Illinois.

a follow-up of less than six months has been included. It is known that 98 per cent of all relapses, in patients infected by this technique and treated with 8-aminoquinolines, occurs before 150 days (unpublished observation). Inasmuch as the variety of toxic manifestations is similar to that of pamaquine, toxicity has been expressed in terms of pamaquine equivalents. All drug was administered orally.

Choice of clinical material. All primary cases treated were characterized by having an incubation period of less than fifteen days. A limited number of patients that had relapsed after treatment with a heterogeneous group of drugs have also been included in this report, but only subjects who relapsed within thirty days after end of therapy were chosen. Craige, et al. (1947) have shown that patients with short prepatent or latent periods offer a severe challenge to curative drugs. Under these experimental

TABLE 4

Curative effect of primaquine when administered together with 1.64 gms. quinine daily in primary attacks of vivax *malaria (Chesson strain)*

CASES	DAYS FROM END OF Rx TO FIRST RELAPSE		FOLLOW UP	RATIO: SUBJECTS RELAPSED/ SUBJECTS TREATED	SYMPTOMS	MET* HGB	MEAN PLASMA† CONCENTRATION OF PRIMAQUINE
	F/102+	Para.					
Daily dose of 22.5 mgm.‡							
			days			*per cent*	*gamma/liter*
1	—	—	370		None	5.3	8
2	—	—	520		None	7.9	—
3	—	—	365		Diarrhea	3.4	10
4	—	—	415		None	6.3	8
5	—	—	364	0/10	None	5.8	5
6	—	—	226		None	4.1	1
7	—	—	341		None	8.7	4
8	—	—	373		None	12.2	9
9	—	—	373		None	8.6	6
10	—	—	389		None	10.3	4

* Expressed as per cent of total hemoglobin (average for last 5 days of treatment).

† Determined by method of Brodie, Udenfriend and Taggart (1947).

‡ Drug administered in 6 divided doses daily for 2 weeks.

conditions the relapse rate after treatment of primary attacks with suppressive drugs approaches 100 per cent (Table 1).

RESULTS

When primaquine was given alone in six divided doses daily, curative effect was demonstrated in doses as low as 22.5 mgm. (base) per day (Table 2).

Subsequent experience with the action of primaquine against trophozoite-induced infections suggests that many, if not all "relapses" reported in Table II were really recrudescences, that is, were due to incomplete eradication of trophozoites, because the parasitemia recurred very early. When primaquine was given in conjunction with 1.64 Gms. quinine (base) daily, considerably greater curative effect resulted (Tables 3, 4, and 5).

A daily dose of 22.5 mgm. of primaquine given concurrently with 1.64 Gms. of quinine (base)[4] prevented relapse in practically 100 per cent of cases. (Tables 4 and

[4] Subsequent studies have shown that 1.64 Gms. of the base is in excess of the amount of quinine

5). Increasing the dose of primaquine to 60 mgm. increased the toxicity without concurrent therapeutic advantage (Table 3).

TABLE 5

Curative effect of primaquine when administered in dosages of 22.5 mgm. with 1.64 gms. quinine daily — in cases representing the first or second relapse after other therapy*

CASES	DAYS FROM END OF Rx TO RELAPSE		FOLLOW UP	RATIO: SUBJECTS RELEASED/ SUBJECTS TREATED	SYMPTOMS	MET† HGB	MEAN PLASMA‡ CONCENTRATION OF PRIMAQUINE
	F/102+	Para.					
			days			*per cent*	*gamma/liter*
First relapses							
1	—	—	755		None	4.6	3
2	—	—	277		None	8.3	9
3	—	—	312		None	5.7	3
4	—	—	330		None	6.0	6
5	—	—	337		Abd. ++	6.5	2
6	—	—	307		None	6.7	2
7	—	—	364	0/13	None	3.4	4
8	—	—	316		None	5.1	3
9	—	—	346		None	8.4	6
10	—	—	343		None	8.1	2
11	—	—	245		None	4.5	—
12	—	—	161		Abd. ++	—	—
13	—	—	285		None	8.3	4
Second relapses							
1	—	—	362		None	4.7	17
2	—	—	369		Abd. +	5.3	13
3	76	74	—		Abd. +	5.6	25
4	—	—	349		None	1.9	5
5	—	—	365	1/8	None	9.3	9
6	—	—	365		Anorexia	7.7	—
7	—	—	349		None	9.4	5
8	—	—	349		Abd. +	7.5	6

* Both drugs were administered in 6 divided doses daily for two weeks.

† Expressed as per cent of total hemoglobin (average for last 5 days of treatment).

‡ Determined by method of Brodie, Udenfriend and Taggart (1947).

Abd. + = mild, transient abdominal cramps.

Abd. ++ = moderate, repeated abdominal cramps.

TOXICITY

Toxicity studies were carried out on volunteers unsuited for therapeutic trials.[5] The drug was given during attacks induced by intravenous malaria. The same drug

needed. A dose of 0.82 Gms. of base (1.0 Gms. quinine sulfate) is certainly sufficient and possibly even as little as 0.547 Gms. of base may suffice. The smallest effective dose of quinine has yet to be determined.

[5] Extensive studies of toxic and therapeutic effect of primaquine in mammals and primates have been done by L. H. Schmidt (Christ Hospital, Cincinnati, Ohio).

TABLE 6

Toxicity studies of Primaquine (SN 13272)

DAYS Rx	CASE	AGE	WEIGHT	SYMPTOMS	LABORATORY FINDINGS	MET* HGB	MEAN PLASMA† CONCENTRATION OF PRIMAQUINE
Daily dose of 120 mgm.‡							
						per cent	*gamma/liter*
14	1	21	145	Abd. ++	Normal	20.5	201
14	2	28	145	Abd. + & Nausea	Normal	21.7	228
14	3	39	168	Abd. +	WC 4700§	18.3	308
14	4	24	150	Abd. ++	Normal	19.6	144
14	5	43	152	Abd. +	Normal	20.7	171
Daily dose of 120 mgm. given concurrently with 1.64 gms. quinine‡							
14	1	36	140	Abd. +++	Normal	9.0	77
14	2	24	172	Abd. +++	Normal	9.8	85
14	3	28	189	Abd. ++ & Nausea	Normal	8.8	132
14	4	39	137	Abd. + & Anorexia	Normal	15.5	126
14	5	30	139	Abd. + & Anorexia	Normal	11.1	74
14	6	22	180	Abd. +++ & Vomiting	Normal	5.5	—

* Expressed as per cent of total hemoglobin (average for last 5 days of treatment).

† Determined by method of Brodie, Udenfriend and Taggart (1947).

‡ All drug given in 6 divided doses daily.

§ 5 per cent immature granulocytes, 18 per cent mature granulocytes, 76 per cent lymphocytes (returned to normal 5 days after last dose of drug).

Abd. + = mild, transient abdominal cramps.

Abd. ++ = moderate, repeated abdominal cramps.

Abd. +++ = severe, persistent abdominal cramps.

TABLE 7

Toxicity studies of Primaquine (SN 13272)

DAYS Rx	CASE	AGE	WEIGHT	SYMPTOMS	LABORATORY FINDINGS	MET* HGB	MEAN PLASMA† CONCENTRATION OF PRIMAQUINE
Daily dose of 240 mgm. given concurrently with 0.199 gms. methylene blue‡							
						per cent	*gamma/liter*
9	1	24	134	Abd. ++	WC 2000§	7.5	395
Daily dose of 240 mgm. given concurrently with 1.64 gms. quinine‡							
11	1	39	160	Abd. ++++	WC 3200 ¶	9.6	213
14	2	36	172	Abd. +++	WC 11,900	10.0	131

* Expressed as per cent of total hemoglobin (average for last 5 days of treatment).

† Determined by method of Brodie, Udenfriend and Taggart (1947).

‡ All drug given in 6 divided doses daily.

§ 13 per cent immature granulocytes, 3 per cent mature granulocytes, 79 per cent lymphocytes (returned to normal 7 days after last dose of drug).

¶ 9 per cent immature granulocytes, 30 per cent mature granulocytes, 56 per cent lymphocytes (returned to normal 14 days after last dose of drug).

Abd. ++ = moderate, repeated abdominal cramps.

Abd. +++ = severe, persistent abdominal cramps.

Abd. ++++ = intolerably severe abdominal cramps.

dosage regimen was followed. The toxic manifestations observed during administration of primaquine at 120 mgm. daily (alone, and in conjunction with other drugs) is shown in Table 6. The toxicity of primaquine tends to be cumulative; in some instances symptoms began late in the course of drug administration and continued for several days after its discontinuance.

Two hundred and forty mgm. (base) probably represents the maximum dose that can be administered with safety for periods longer than a week even under close observation in hospital (Table 7). Although toxic manifestations were severe, no irreversible damage was noted. In contrast, the maximum tolerated dose of pamaquine is probably 90 mgm. (base) per day; and, for pentaquine (SN 13,276) is 120 mgm., but severe damage to the nervous system may result from its administration at that dose. Of the curative anti-malarial drugs extensively studied, only isopentaquine (SN 13,274) can be given safely at a dose of 240 mgm. (base) per day for an extended period.

It is of interest to note the effect of quinine on the production of methemoglobin. At high doses of primaquine with quinine the methemoglobin is roughly 50 per cent as great as that formed by the same dose of primaquine given alone (Table 6).

DISCUSSION

The therapeutic significance of the change in character of the terminal amino groups on the side chain of pamaquine-like compounds can be seen by the following comparison. (For greater homogeneity of data only primary cases with a standardized infection are summarized):

DRUG*	DAILY DOSE† (BASE WEIGHT)	RELAPSE RATIO	ESTIMATED DOSE FOR 100% CURE
	mgm.		*mgm.*
Pamaquine	60‡	6/10	90–120
Isopentaquine	60	3/10	90
Primaquine	22.5	0/10	22.5

* Administered concurrently with quinine.

† Given in 6 divided doses for 2 weeks.

‡ This is equal to 133 mgm. of the salt, pamaquine naphthoate.

It is apparent that on an equal weight basis, primaquine is about four times as active as the best of the other members of the family. Comparison of the subjective toxicity in the pamaquine family in terms of estimated pamaquine equivalents are as follows:

DRUG	TOXICITY		CHEMO-THERAPEUTIC* INDEX
	In terms of symptomatology at 60 mgm./day	In terms of maximum tolerated dose	
Pamaquine	1.00	1.00	1
Isopentaquine	0.75	0.33	2½
Primaquine	1.00	0.33	10

* Chemotherapeutic index is the ratio of largest tolerated dose divided by the smallest dose capable of preventing nearly all relapses.

Although not the subject of this paper, which stresses comparative curative effect and toxicity of the three drugs studied under standard conditions, it should be mentioned further that primaquine can establish a high prophylactic and curative ratio when administered in therapeutically safe single daily doses. This is not possible with either pamaquine, pentaquine or isopentaquine. These latter three drugs have been shown in field studies to be active in doses one-half to one-third as great as those necessary to produce equivalent results against our standard test strain of *vivax* malaria (Most, et al., 1946), (Alving, 1948), (Coggeshall and Rice, 1949). Observation of a limited number of patients suggests that naturally acquired infections likewise can be cured with much smaller doses of primaquine than reported here.

Primaquine is superior to both pamaquine and isopentaquine because it will cure severe infections of *vivax* malaria in dosages that are relatively non-toxic in white subjects and because it has a wide range between the clinically effective dose and the maximum tolerated dose.

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A MALARIAL PARASITE OF THE AFRICAN ELEPHANT SHREW, *ELEPHANTULUS RUFESCENS DUNDASI* DOLLMAN[1]

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Malarial parasites are known to occur naturally in only a few groups of the mammals of the world. One of them was described in 1913 from elephant shrews in the Belgian Congo. In the hope of learning more about this parasite and possibly of obtaining a strain for laboratory study Huff requested that members of the 1948 Naval Medical Science Group accompanying the University of California African Expedition search for infected elephant shrews, and Hoogstraal and Lawless were assigned as a field team to accomplish the task. Infected elephant shrews were easily found at Kapoeta in southeastern Anglo-Egyptian Sudan, and after an initial period of domestication of the host and field observations, 104 living elephant shrews, believed to the the first to reach the States, were flown back to the Naval Medical Research Institute for further investigation. As a result of the 1948 findings, the field team was sent back to the Sudan in 1949–1950 under the auspices of the U. S. Naval Medical Research Unit No. 3, but before the mission could be satisfactorily completed a change in the overall program of the Unit made it necessary to discontinue the project. The known facts about the elephant shrew organism are now presented in the hope of stimulating further research on this subject.

A malaria-like parasite was first described by Rodhain, Pons, Vandenbranden and Bequaert (1913) from five giant elephant shrews, *Petrodromus tetradactylus* Peters between Sankisia and Bukama, in the Belgian Congo. Descriptions of sexual forms and of what were presumed to be asexual forms of the parasite were given, and the name, *Plasmodium brodeni*, was proposed by these authors. The brief description of the sexual forms agrees well with the parasites we have recovered from the small elephant shrew, *Elephantulus rufescens dundasi* Dollman, in the Southeastern Anglo-Egyptian Sudan.

We have also seen what might appear to be asexual forms among our Sudan speci-

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The opinions and statements contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

mens but in each case they proved to be multiple infections of the red blood cell with immature gametocytes, and we strongly suspect that this is what the workers in the Congo saw.

A mention of elephant shrew malaria from *Petrodromus venustus* Thomas from Nyasaland is found in Bruce, Watson, Hamerton, and Bruce (1915). The reference indicates that the parasites were common in this species of host and bore a resemblance to the gametes seen in the blood of monkeys. The four figures in color are all of gametocytes but they do not contain enough detail upon which to base a comparison with the parasite we have studied from the Sudan elephant shrew.

Until the questions of the presence or absence of exoerythrocytic schizogony and of the transmission of malaria-like organisms in mammals are answered the exact evolutionary relationships and taxonomic status of this group must remain in doubt. Finding the answers for one species will probably provide the key for working with species in other mammalian hosts, and, we feel, will considerably assist in explaining the still poorly understood exoerythrocytic cycle of human malarias.

OBSERVATIONS ON *PLASMODIUM BRODENI*

(a) *Description of Erythrocytic Forms.—Location collected:* Torit, Ikoto, and Kapoeta, Equatoria Province, Anglo-Egyptian Sudan. *Host: Elephantulus rufescens dundasi* Dollman. *Parasite size range:* The most immature signet ring forms measure from one to two microns in diameter. Mature gametocytes measure 9 to 10 microns. Host erythrocytes measure from 8 to 10 microns.

Developmental Forms: Only immature, developing and mature male and female gametocytes have been noted in the peripheral blood. In several instances, multiple infections of mature and immature gametocytes have given the appearance of early segmenters.

Description of Parasite: The following description is based on observations of infections from over 500 hosts taken at all seasons. The staining was all with Giemsa.

Developing forms

The most immature forms, measuring from 1.5 to 2.0 microns in diameter, contain a single compact nucleated mass of red chromatin material surrounded by a compact area of non-pigmented blue cytoplasm. As development progresses this restricted mass of cytoplasm loses its compact appearance and a vacuole appears, thus creating a ring of blue, non-pigmented cytoplasm interrupted by a small red mass of chromatin. The compact nuclear mass of the very young gametocyte often contains a central, clear, unstained nuclear mass. As the parasite reaches about one-third its maximum size the compact nucleus assumes an irregular, elongated, or comma shape, and individual dark red chromatin granules become more apparent. No host cell enlargement or loss of hemoglobin is observed at the one-third grown stage unless the cell is multiply infected. We have observed up to 12 parasites of this stage in a single cell, in which case it was enlarged to the extent of 1 to 4 microns.

A definite change in the parasite becomes apparent as it passes about one-third its maximum size. The cytoplasm begins to thicken, becomes slightly granular, and a slight amoeboid action may be observed. At this stage, slender, irregular projec-

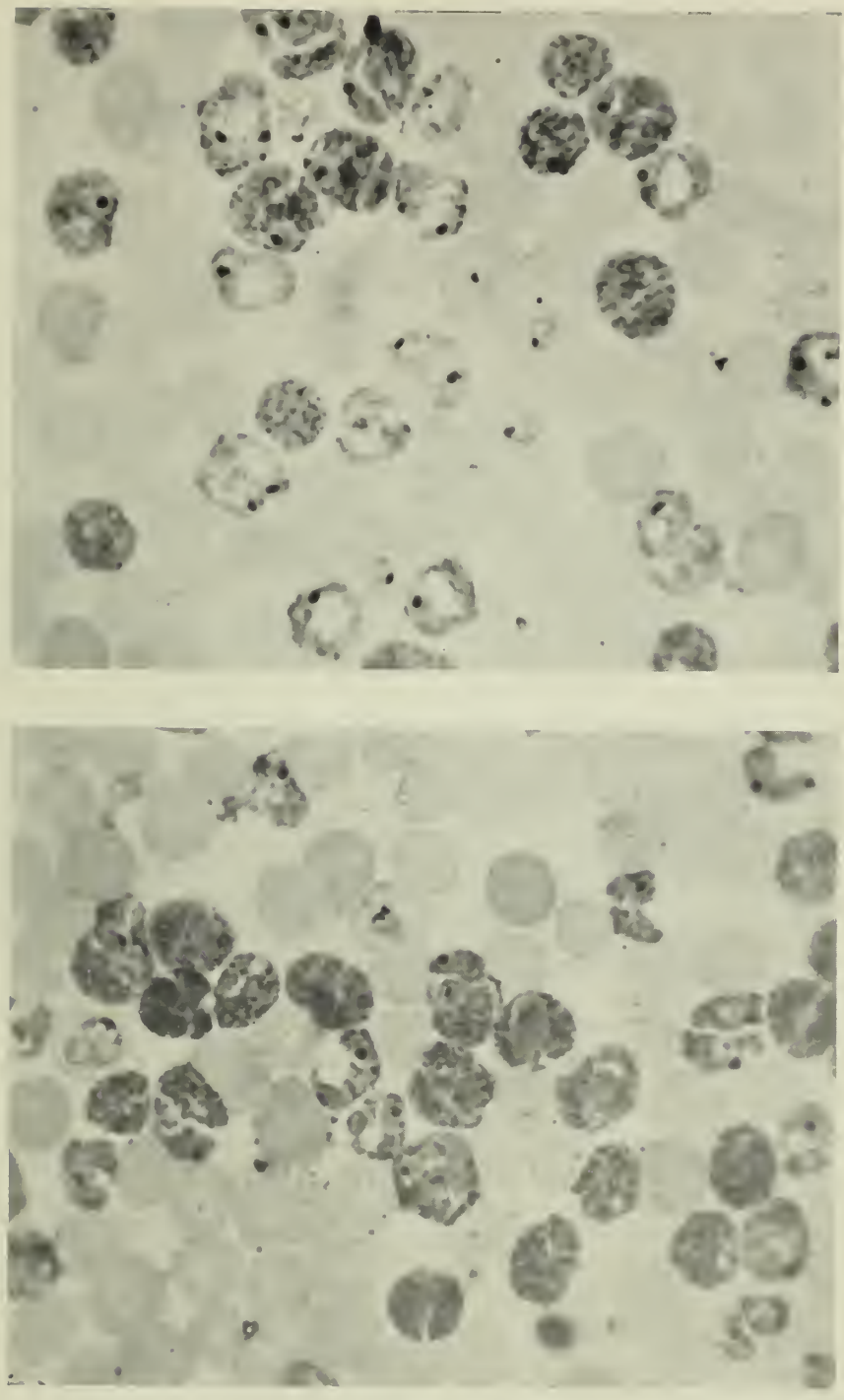

FIG. 1. Blood smears from natural infections of *Plasmodium brodeni* in *Elephantulus rufescens dundasi* from Southeastern Anglo-Egyptian Sudan. Several ring-like, young gametocytes are shown in the photograph above. Single, double, and multiple infections of host cells are abundant.

tions of cytoplasm, additional vacuolated areas, and fine, evenly distributed pigment granules begin to appear. As growth continues the periphery becomes coarser and more ragged, and the cytoplasm often appears paler blue because of the increased vacuolation. A slight loss of hemoglobin may now be noted in the host cell but no cell enlargement appears unless three or four parasites have invaded it.

When the parasite is three-fourths grown it occupies much or most of the host cell, and little or no hemoglobin is apparent. The parasite is compact and usually round in outline, but may be ragged or slightly amoeboid. Fine, blackish pigment granules are evenly distributed throughout the medium blue cytoplasm, and at least two vacuoles are usually present. The nucleus is 2.5 to 3 microns in diameter, and usually appears granular. Conspicuous chromatin particles are confined within a

FIG. 2. *Elephantulus rufescens dundasi* Dollman. (Official U. S. Navy photograph by Harley J. Cope).

rather indistinctly demarcated nuclear sphere whose background is paler than the darker red chromatin particles.

The exact point at which developing gametocytes begin to differentiate themselves into micro- and macrogametocytes is indefinite. Mature gametocytes are easily differentiated from one another.

The Microgametocyte: The individual microgametocyte is usually amoeboid and often bizzare in shape, very rarely round or oval. At least one irregular vacuole (sometimes two) is always visible within the irregular grayish yellow brown cytoplasmic pattern. The brilliantly staining eccentrically placed nuclear sphere consists of a compact medium to dark red staining nucleolus surrounded by a pink red halo, clearly delimited from the cytoplasm of the parasite. The nuclear sphere often fragments into the cytoplasm of the parasite. Golden to dark yellow brown pigment particles are finely and regularly distributed throughout the cytoplasm.

The Macrogametocyte: The macrogametocyte is a round, or ovoid, compact cell, often one-quarter larger than the normal red cell of the host. Its cytoplasm is dense and dark blue (rather than yellow brown as in the microgametocyte), and its dark brown to black pigment particles are more coarse and clumped. There is generally little or no vacuolation within the cytoplasm. The pigment particles are so coarse and clumped that the cytoplasm appears "dirty." The nuclear wall is not apparent. A circle or oval of regularly arranged dark red chromatin particles separates the nuclear area from the cytoplasm. The nuclear background stains pale red and contains dark red chromatin particles. The nucleus is situated in or closely adjacent to the center of the parasite.

Red blood cells containing both a macro- and a microgametocyte are rarely seen except in heavy infections, in which they are not uncommon.

Developmental Cycle in Peripheral Blood: We have never been fortunate enough to find a host showing only immature stages in the peripheral blood. However, in animals showing a large percentage of very young forms we have watched a gradual development into a large percentage of mature forms requiring a 50 hour period. Towards the end of this period very young forms began to appear again in the blood. The proportion of male and female gametocytes varies considerably though we observed very few individual cases in which there were more micro- than macrogametocytes. Our averages range from between 20% to 110% more macrogametocytes than microgametocytes.

We have seen cases in which 60% of the hosts's red blood cells were parasitized and multiple infections of the cell were the rule rather than the exception. Infections in which 10 or 20% of the red cells were parasitized were not infrequent during the wet season.

(b) *Exflagellation.*—On two occasions fresh blood from heavily infected elephant shrews was placed in a moist chamber and observed at fifteen minute intervals for one hour. No exflagellation was demonstrated.

(c) *Transmission and Vector Problems.*—Numerous attempts by a number of workers to find the natural vector or experimental vectors of the group of malaria-like organisms of mammals which produce no schizogony in the blood have failed entirely or have given inconclusive results, and the question is still in the theoretical stage (Huff 1945).

In considering all biting arthropods which we might have to study in relation to the elephant shrew organism, we have had reason to dismiss some immediately. Fleas and lice are almost or completely absent on the Sudan elephant shrews and in the holes in which they sometimes live. Simuliid flies are absent from the areas in which we collected; streams in which they breed are many miles distant from where the animals were taken. We have found so few Reduviid bugs that we are inclined to dismiss them as possible vectors. Both Hippoboscid flies and Argasid ticks might feed and move away, but we have found no Argasid ticks in the particular areas worked, and no evidence that the winged avian or wingless, antelope Hippoboscid flies feed on other than their specific hosts.

We had hoped to experiment with *Flebotomus* in spite of some circumstantial evidence against this group as possible vectors. *Flebotomus* does breed during the

dry season in the deep cracks of the cotton soil of the Southeastern Sudan, and we should have expected to find a higher rate of infection in elephant shrews during the dry season if these flies had been involved.

Many species of Tabanid flies and some of *Glossina* are very common throughout the range of the elephant shrews and our work with them was just commencing when the project was terminated.

Larval and nymphal ticks, *Rhipicephalus evertsi* Neumann, were exceedingly common on almost every animal examined at all times of the year (the original authors of *P. brodeni* also mention immature ticks on their elephant shrews). The larvae were on the edges of the ears and the nymphs were almost anywhere but especially on the undersides and on the penal sheath of the males. Dissection of numerous nymphs taken from infected animals failed to reveal anything other than deteriorating parasites in the ingested blood. We were, however, sufficiently impressed with the possibility of their being vectors that we had started a colony of this tick before we were recalled to Cairo.

Exceedingly minute mites were not infrequently found about the anus of the elephant shrew, but mites were not found in the fur. We had made no attempt to test the possible rôle as vectors of this minute species.

Mosquitoes, of course, strongly suggest themselves in spite of many failures to incriminate them in other species of malaria-like infections of mammals. In 1948 we unsuccessfully attempted to infect the following species of mosquitoes breeding in close proximity to the hiding and foraging places of the elephant shrew: (Species bred in active colonies in numbers over 300): *Eretmapodites silvestris conchobius* Edwards, *Anopheles* (*Myzomyia*) *gambiae* Giles, and *Culex* (*Culex*) *poicilipes* Theobald; (Species bred in small numbers or so inactive as to be hardly worthy of consideration as a fair trial): *Aedes* (*Diceromyia*) *furcifer* (Edwards), *A.* (*Aedimorphus*) *cumminsi* (Theobald), *A.* (*Aedim.*) *ochraceus* (Theobald), *A.* (*Stegomyia*) *simpsoni* (Theobald), *Culex* (*Lutzia*) *tigripes* Grand. and Char., *C.* (*Culiciomyia*) *nebulosus* Theobald, and *C.* (*Neoculex*) sp. nov.

The following species of mosquitoes were fed upon the most heavily infected elephant shrews among those which were transported by airplane to the Naval Medical Research Institute in 1948. No malarial oocysts were observed upon dissection.

SPECIES OF MOSQUITO	NUMBER OF DISSECTIONS
Anopheles quadrimaculatus Say	68
Anopheles albimanus Wiedemann	41
Anopheles freeborni Aitken	23
Aedes aegypti Linnaeus	58
Aedes triseriatus Say	63
Culex pipiens Linnaeus	16
Culex quinquefasciatus Say	5
Total	274

(d) *Search for Exoerythrocytic Stages.*—Since all attempts to find the vectors of the malaria in the elephant shrews were unsuccessful it was impossible to prepare

material for examination for pre-erythrocytic stages. Intensive study, however, was made of the tissues of the naturally infected, individual shrews in the blood of which large numbers of gametocytes or early stages in the development of gametocytes were found. The organs were fixed in zenker-formalin fixative, prepared and imbedded in celluloidin, sectioned, and stained by the hematoxylin eosin-agar method used by Huff and collaborators in their studies of the exoerythrocytic stages of various malarial parasites (for details, see Huff and Coulston 1944). By these methods sections were made of the spleen, liver, bone marrow, tongue and skeletal muscle, pancreas, brain, testis, intestine, kidney, lung, eye, and heart. Much time was given to the study of these organs and especial attention was given to the liver and spleen.

No evidence of any exoerythrocytic stages was found. Phagocytosis of erythrocytic stages was observed in the liver and spleen and large amounts of melanin were observed in the macrophages of these organs. Since the entire organs were sectioned and all of the sections examined in the case of the small organs like spleen, and sections were made through the entire organ in the case of the larger organs like heart and liver, the chances of discovering any possible exoerythrocytic stages were good.

(e) *Seasonal Periodicity.*—The incidence of infection among the elephant shrews and the average density of infection varied in direct relation with the season. We have observed 100% infection at the height of the rainy season (August), a reduction to 60% early in the dry season (November), further dry season reductions to 35% in December and 25% in February, and gains to 85% early in the rainy season (May). From 46 to 90 elephant shrews were used in each of these samples. Between December and March (dry season) immature stages of the parasite were seen in only 2% of the infections, but during the rainy season they could be found in almost every specimen, indicating, we should think, rather frequent innoculations by the vector during this period.

In each of our dry season samples we have found one or two hosts with heavy infections (more than one parasite per field), while almost all the others had low infections (less than three parasites per ten fields). We are unable to say whether these rare heavy dry-season densities are due to new innoculations or to relapses.

(f) Attempts were made to transfer the infection from heavily parasitized shrews to domestic rats and to shrews of the same species the blood of which was negative. These attempts met with failure which is additional evidence favoring the belief that asexual stages were absent from the blood of the donor.

OBSERVATIONS OF THE HOST

Our experience with the host, *Elephantulus rufescens dundasi* Dollman, has already been fully presented (Hoogstraal 1950). Elephant shrews are African insectivores of the family Macroscelididae. Of the seven genera, one other, *Petrodromus*, has been mentioned as a host of this or a similar species of malaria-like organism. The genus *Elephantulus* comprises several species with about thirty forms, and ranges from the Cape to Algeria and into Asia Minor. Most, if not all of the species appear to prefer the somewhat drier and unforested parts of the continent. *Petrodromus*, consisting of about a dozen forms, ranges throughout East, Central, and South Africa, and most of its forms appear to prefer more heavily vegetated habitats.

Ants and termites are the favorite food of the species we studied, though other small insects and a small amount of vegetable matter are also consumed. Some species characteristically dig holes but the Sudanese one, at least, seldom does. It lives in small patches of more or less dense vegetation in grassland, and hides by day near tree trunks or among plant debris or undergrowth. It is active at all hours of the day and night, but more so at night and in early morning and late afternoon and evening. One or two young are born at a time, fully developed, at any season of the year. Adults are the size of a small rat, and can run or jump with equal ease.

These animals have never been known to breed in captivity, and young born shortly after capture are invariably destroyed by the mother, though young brought in with the mother are often well cared for in the cage. Dr. C. J. van der Horst of the University of the Witwaterstrand at Johannesburg, who has been trying to breed these animals in captivity for many years, suggests that if American workers wish to make an attempt to breed them for experimental purposes, they be transported to Southern California and placed in large, open-air, secluded pens. Numerous closely confined elephant shrews show hysterogenic tendencies and more than a pair should never be caged together. All food given captive animals must be finely ground or in very small pieces, and the properties must nearly equal that of their natural food. Live insects are the most satisfactory sustenance. Little success can be anticipated in keeping captive animals alive for any length of time if they are not carefully housed and fed.

Van der Horst (1946) considers the natural life span of a South African species of *Elephantulus* to be thirteen months, and states that in exceptional circumstances individuals may survive nineteen months.

Although the 104 elephant shrews sent back to Bethesda by air from the Sudan were subjected to extreme heat on some of the stops during the five day drip and to cold temperatures at other times, none died en route and only one already injured individual died soon after arrival. Live insects were not provided until late during the third day when grasshoppers were obtained. This lot was one that had been in captivity for from two to four weeks with almost no deaths and had been extremely carefully cared for. We cite this information, which does not appear in our published account of the elephant shrew, to show that with proper care these animals can be kept in captivity and transported long distances, when need be. They are, however, not a satisfactory laboratory animal in the general sense of the term.

DISCUSSION

(a) *Comparison of Sudanese Material with the Species Described from Petrodromus tetradactylus.*—So far as the original description of *Plasmodium brodeni* from *Petrodromus tetradactylus* in the Belgian Congo goes, we can find only one real difference between it and the material found by us in *Elephantulus rufescens dundasi* of the Southeastern Anglo-Egyptian Sudan. The original authors of *P. brodeni* describe only mature gametocytes and mention immature individuals simply in that "a strand of globular cytoplasm borders one side of the parasite." The size of the specimens from both areas is essentially the same, and, allowing for slight differences in stain,

the colors are similar. The chief difference, in so far as we can see, is that Rodhain *et al* state that the pigment in the microgametocyte is less uniformly distributed than in the macrogametocyte. In the Sudan material we observe that in the microgametocyte the pigment particles are finely and regularly distributed throughout the cytoplasm and in the macrogametocyte the pigment particles are coarse and clumped. We do not feel, however, that at this time we can classify our material as a separate species on the basis of this single difference. The general characteristics of vacuolation and of nuclear formation and position appear to be similar in the Congo and Sudan materials.

As for the asexual forms mentioned in the original description of *P. brodeni*, we feel confident, as stated earlier in this paper, that they represented multiple infections of the red blood cell with immature gametocytes. We made the same mistake during the first phase of our work, but by careful and repeated observations on a considerable amount of material satisfied ourselves on this point.

(b) *Transmission Problems.*—We cannot overemphasize the need at this stage of knowledge of mammal malarias for finding their vectors for purposes of life history studies and for determining the systematic position of the species involved. Huff (1945 and 1947) has already stressed this need and discussed the implications involved. He believes that the malarial parasites have evolved through the diptera or their progenitors and that eventually the evolutionary and systematic position of the vector will be shown to parallel that of the malarial parasite found in the vertebrate. The vertebrate infection, with which we are of necessity most familiar, is biologically little more than a secondary infection. We believe that this reasoning must be more fully investigated before we can consider that we are familiar enough with the life cycle of human and other mammalian malarias to place them in their proper systematic categories.

The only lead we have towards involving an insect vector is the claim by Mer and Goldblum (1947) that they found sporozoites in one specimen of a Nycteribiid fly taken from bats infected with malarial parasites. Bat malarial parasites are similar to *P. brodeni* in lacking erythrocytic schizogony. This gives some indication that Pupipara might be involved as vectors of bat malaria at least, and the close association of this group of flies with many bats provides strong circumstantial evidence in that direction. After no little investigation of the possible association of Pupipara and elephant shrews in the Sudan, we were forced to conclude that there was no apparent relation between the two, but the matter is open to further research. Tsetse flies, *Glossina* spp., which are closely related to the Pupipara and similar in many respects, we considered much more possible vectors but we did not have the opportunity to study them before we left the Sudan.

Various unsuccessful attempts have been made to infect mosquitoes with some of the lower mammalian malarias. To one familiar with the trials and tribulations of maintaining a vigorous colony of many wild mosquitoes through successive generations under tropical conditions it does not appear that these trials have been exhaustive enough to definitely eliminate mosquitoes as vectors, in spite of the fact that Garnham (1948B) does consider that they may be rejected. We still feel that attempts to transmit malarias which do not possess erythrocytic schizogony should

be made with large quantities of local mosquitoes, at optimum conditions and at all stages of the infection.

The marked and direct correlation of the incidence and density of the infection in the erythrocytes of the host with the dry and wet seasons appears to indicate that the vector is largely or wholly a rainy season arthropod. The rare infections with young gametocytes that we found during the dry season we attribute to relapses or to chance infection by out-of-season vectors. The fact that mature gametocytes can be found in a small percentage of elephant shrews throughout the dry season seems to indicate that some of these forms are long-lived in the peripheral blood. We should think that if relapses were to account for these latter, we would have found a higher percentage of immature gametocytes.

Young elephant shrews are born the year around and the habits of the animals show little change, except that they range somewhat farther afield in the tall grass that grows up during the rains than they do during the dry season. From this we conclude that the life history of the host is hardly a factor in the seasonal periodicity of the infection.

(c) *Exoerythrocytic Stages.*—Considering the abundance of exoerythrocytic stages of *P. kochi* and *P. cynomolgi* of monkeys reported by Garnham (1948A) and Shortt, Garnham and Malamos (1948) respectively, it may seem surprising that we were unable to discover the exoerythrocytic stage of *P. brodeni*. However, we can have no *a priori* assurance that the morphology, distribution, or prevalence of the exoerythrocytic stages of *P. brodeni* would be like those of the simian parasites. Attention has recently been called to the extreme difficulty of finding pre-erythrocytic stages in infections such as *P. gallinaceum* in chicks even after the inoculation of the sporozoites from 200–300 heavily infected mosquitoes (Huff 1950). If the hypothetical exoerythrocytic stages of *P. brodeni* should be primarily pre-erythrocytic we should expect them to be extremely difficult to demonstrate in naturally acquired infections in which it is only reasonable to assume that a comparatively small number of infected vectors were involved in the transmission. Considering the extremely heavy parasitemia of the elephant shrews which were killed for tissue study (some of them with 25 gametocytes per microscopic field) we may be reasonably sure that the exoerythrocytic stages are rare when compared with such infections as *P. gallinaceum*.

(d) *Taxonomic Status and Relation to other Mammalian Malarias.*—The relationship of *P. brodeni* to other mammalian malarial parasites which are similar to it in having no known erythrocytic schizogony is of considerable interest. Garnham (1948A) has described a very spectacular type of exoerythrocytic development of *P. kochi* characterized by large merocysts up to 2 mm. in diameter in the liver of infected monkeys. He believed that the exoerythrocytic stages persist during the course of the parasitemia and continue to give rise to the gametocytes in the blood. Similar types of development were described by Field and Edeson (1949) for *P. vassali* of a Malayan squirrl and by Ray (1949) for an undescribed parasite from a Himalayan flying squirrel. It would appear to be unlikely that a development similar to that described in these three parasites occurs in the infections of elephant shrews with *P. brodeni* for merocysts of the dimensions found in the former would probably not have

been missed in the very thorough search which we made of the tissues of heavily infected animals.

The descriptions of structures supposed to be exoerythrocytic stages in the malaria of bats appear to place them in a different category from the ones described from *P. kochi*, *P. vassali* and the *Plasmodium* from the Himalayan flying squirrel. Manwell (1946) described unpigmented schizonts from the blood of a fruit bat (*Pteropus gouldi*) and Mer and Goldblum (1947) described schizogony in smears of bone marrow, lung kidney and liver of *Myotis myotis*. The latter observers described schizonts in macrophages, granulocytes, and "reticulum cells." Both the morphology and the host cell preferences of these bat parasites differ from the descriptions of large hepatic merocysts in the above mentioned species.

Although the erythrocytic stages of the parasite under discussion bear a closer resemblance to the gametocytes of *Plasmodium* than to the corresponding stages of *Haemoproteus* or *Leucocytozoon* the absence of erythrocytic schizogony suggests the possibility that this species may be more closely related to the Haemoproteidae than to the Plasmodidae. Manwell (1946) has suggested that the malarial parasites of Chiroptera may need to be placed in a new genus with characters somewhat intermediate to those of Haemoproteidae and Plasmodidae. Garnham (1948A & B), on basis of his study of the exoerythrocytic development of *P. kochi*, has placed this species in the genus *Hepatocystes* Levaditi and Shoen, 1932.

Some revision of the Sub-Order Haemosporidiidea (Order Coccidiida) into which all of the known malarial and malaria-like parasites are placed is certainly needed. However, before the genus *Hepatocytes* can be recognized, or new genera can be proposed, or the known species can be reassigned to these genera it would appear that many points in the life-cycles of several of the species must be clarified. The systematic position of vectors is, at present, the best guide we have to the systematic relationships of their respective parasites. As yet, the vectors of *P. kochi*, *P. vassali*, *P. brodeni*, and the malarial parasites of bats are unknown. The distribution of the various stages of the parasites among the various cells of the blood or tissues of the host is an unreliable guide to the systematic relationships of the parasites. We need only reflect on the wide variations that exist in the various known exoerythrocytic stages of several species of *Plasmodium* to set this fact into evidence. Furthermore, the presence or absence of schizogonic forms in the blood cells as a criterion is subject to some confusion since some species of *Plasmodium* (e.g., *falciparum* and *mexicanum*) at times apparently lack erythrocytic schizonts. Garnham's (1948A) emendation of *Hepatocystes* Levaditi and Schoen, 1932 based upon the type and location of the exoerythrocytic stages appears premature for the following reasons: (1) Our knowledge of the morphology and distribution of the exoerythrocytic stages of related species is not sufficient to permit setting up the characteristics of a new genus; (2) the emended genus is restricted to parasites of lower African monkeys which develop in the parenchyma of the liver; (3) other characteristics now unknown such as the vectors may prove to be the more reliable criterion for the erection of genera in this group of organisms.

We recommend that the malarial parasite of the elephant shrew described as *Plasmodium brodeni* Rodhain, Pons, Vandenbranden, and Bequaert, 1913, be re-

tained in the genus *Plasmodium* until the characteristics necessary to an accurate allocation to genus have been discovered.

SUMMARY

1. Field and laboratory studies were made on the malarial parasites of more than 500 specimens of the elephant shrew, *Elephantulus rufescens dundasi* Dollman, from Torit, Ikoto, and Kapoeta, Equatoria Province, Anglo-Egyptian Sudan.

2. The parasites largely agree with the description of *Plasmodium brodeni* Rodhain, Pons, Vandenbranden, and Bequaert (1913) from giant elephant shrews, *Petrodromus tetradactylus* Peters, taken between Sankisia and Bukama in the Belgian Congo, except that no asexual forms were discovered. It is believed that the forms described by Rodhain *et al.* as asexual were multiple infections with immature gametocytes.

3. The arthropods which were considered as possible vectors because of their presence in the area occupied by the elephant shrew or their close relationship with the animal were the species of mosquitoes mentioned in paragraph 4 of this summary, larval and nymphal ticks (*Rhipicephalus evertsi* Neumann), various species of Tabanids and tsetse flies, and possibly but not probably *Flebotomus*.

4. Negative results were obtained in attempts to infect the following mosquitoes breeding in close proximity to the shrews: *Eretmapodites silvestris conchobius* Edwards, *Anopheles gambiae* Giles, *Culex poicilipes* Theobald, *Aedes furcifer* Edwards, *A. cumminsi* Theobald, *A. ochraceus* Theobald, *A. simpsoni* Theobald, *Culex tigripes* Grand. and Char., *C. nebulosus* Theobald, and *C.* sp. nov.

5. Negative results were also obtained from the following laboratory mosquitoes fed upon heavily infected shrews which had been transported to the Naval Medical Research Institute: *Anopheles quadrimaculatus* Say, *A. albimanus* Wiedemann, *A. freeborni* Aitken, *Aedes aegypti* Linnaeus, *A. triseriatus* Say, *Culex pipiens* Linnaeus and *Culex quinquefasciatus* Say.

6. Attempts to transmit the infection from infected shrews to domestic rats and to uninfected shrews of the same species met with failure.

7. Intensive study of sections of spleen, liver, bone marrow, tongue, skeletal muscle, pancreas, brain, testis, intestine, kidney, lung, eye, and heart of heavily infected shrews failed to reveal any evidence of exoerythrocytic stages.

8. Variations in incidence of infection in the shrews were correlated to seasonal changes, the incidence was highest (100%) at the height of the rainy season (August), declined (to 60%) during the dry season (November), continued to decline (to 25%) during the dry season, and increased (85%) early in the rainy season (May). Young gametocytes were most prevalent during the rainy season.

9. The short-lived elephant shrew is difficult to domesticate in numbers, and produces few offspring in nature and none in captivity, so it can hardly be considered a suitable laboratory animal.

10. Although *P. brodeni* would appear to be related to bat malaria, to *P. vassali* of the squirrel, and to *P. kochi* of African monkeys because of the absence of erythrocytic schizogony the true relationship among these species will not be understood until

their vectors and the developmental cycles in the vertebrate hosts are more completely known.

11. It is believed that any revision of the Sub-Order Haemosporidiidae to which all malarial parasites are now assigned should await more complete information on the vectors and life-cycles of the various species.

SUMARIO

1. Se hicieron estudios de campo y laboratorio con parásitos maláricos de más de 500 especímenes de la serpiente elefante, *Elephantulus rufescens dundasi* Dollman, de Torit, Ikoto y Kapoeta, Provincia de Equatoria, Sudán Anglo-Egipcio.

2. Los parásitos corresponden grandemente a la descripción del *Plasmodium brodeni* Rodhain, Pons, Vandenbranden y Bequaert (1913) de la serpiente gigante elefante, *Petrodomus tetradactylus* Peters obtenidas entre Sankisia y Bukama en el Congo Belga, con la excepción de que no se descubrieron formas asexuales. Se cree que las formas descritas por Rodhain *et al.* como asexuales fueron infecciones múltiples con gametocitos jóvenes.

3. Los artrópodos considerados como posibles vectores debido a su presencia en el área ocupada por la serpiente elefante o su estrecha relación con el animal fueron las especies de mosquitos que se mencionan en el párrafo 4 de este sumario, larvas y ninfas de garrapatas (*Rhipicephalus evertsi* Neumann), varias especies de Tabanidos, moscas tse-tse y posible, pero no probablemente *Phlebotomus*.

4. Se obtuvieron resultados negativos en los intentos de infectar los siguientes mosquitos q. viven en la proximidad de las serpientes: *Eretmapodites silvestris conchobius* Edwards, *Anopheles gambiae* Giles, *Culex poicilipes* Theobald, *Aedes furcifer* Edwards, *A. cumminsi* Thebald, *A. ochraceus* Theobald, *A. simpsoni* Theobald, *Culex tigripes* Grand y Char., *C. nebulosus* Theobald, *C. Sp. nov.*

5. También se obtuvieron resultados negativos con los siguientes mosquitos alimentados en el laboratorio en serpientes altamente infectadas transportadas al Instituto de Investigaciónes Médicas de la Armada: *Anopheles quadrimaculatus* Say, *A. albimanus* Wiedeman, *A. freeborni* Aitken, *Aedes aegypti* Linnaeus, *A. triseratus* Say, *Culex pipiens* Linnaeus y *Culex quinquefasciatus* Say.

6. Estudios intensivos de cortes de bazo, hígado, médula de los huesos, lengua, músculos del esqueleto, pancreas, cerebro, testículos, intestino, riñón, pulmón, ojo y corazón de serpientes altamente infectadas no mostraron evidencia alguna de estados exoeritrocíticos.

7. Se correlacionaron las variaciones de la incidencia de la infección con cambios estacionales y se notó que la incidencia fué mayor (100 por ciento) al máximum de la estación lluviosa (agosto), bajó (a 60 por ciento) durante la estación seca (noviembre), continuó declinando (a 25 por ciento) en esta misma estación aumentó (a 85 por ciento) al comienzo de la estación lluviosa (mayo). Gametocitos jóvenes eran más abundantes durante la estación lluviosa.

8. La serpiente elefante es de corta vida y difícil domesticarla en grandes cantidades. Se reproduce poco en la naturaleza y no lo hace en cautividad, por lo que no puede ser considerada como un animal conveniente para el laboratorio.

9. Aunque *P. brodeni* parece vecino a los parásitos de la malaria de los murciélagos, a *P. vassali* de las ardillas y a *P. kochi* de los monos africanos por la ausencia de esquizogonia eritrocítica, la verdadera relación entre estas especies no se entenderá hasta que sus vectores y sus ciclos de desarrollo en los huéspedes vertebrados no se conozcan en mayor detalle.

10. Se cree que cualquier revisión del Sub-Orden Haemosporidiidae en el cual se incluyen hoy todos los parásitos maláricos requiere una información más completa en cuanto a vectores y ciclos evolutivos de las diferentes especies.

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EXPERIMENTAL INFECTIONS WITH *PLASMODIUM FALLAX* SCHWETZ ISOLATED FROM THE UGANDA TUFTED GUINEA FOWL *NUMIDA MELEAGRIS MAJOR* HARTLAUB[1]

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Since Schwetz (1930) described *Plasmodium fallax* from an owl (*Syrnium nuchale*) from the Stanleyville region of the Belgian Congo, no further records have come to our attention, and apparently the species was never isolated in laboratory animals. The following is a description of experiments upon a species of malaria parasite, believed to be identical with *P. fallax*, isolated from an African guinea fowl from the Anglo-Egyptian Sudan. Its course of infection in pigeons, its vectors, and its pre-erythrocytic stages are here described.

EXPERIMENTAL

Isolation. Among other animals obtained by the Naval Medical Science Group of the University of California African Expedition and shipped to the Naval Medical Research Institute were two Uganda tufted guinea fowl (*Numida meleagris major* Hartlaub) which were received in October of 1948. These birds were caught seven miles east of Torit (village of Labalwa), Equatoria Province, Anglo-Egyptian Sudan. One of these specimens (A) was infected with young and mature gametocytes of *Haemoproteus*, gametocytes of *Plasmodium sp.*, *Leucocytozoon*, and an erythrocytic parasite resembling *Aegyptionella*. The other (B) was infected with *Leucocytozoon* and what appeared to be a typical *Haemoproteus*. We did not succeed in isolating anything by subinoculation of blood from specimen A. However, subinoculations of blood from specimen B into chicks was followed by slight parasitemia in one of the chicks on three successive days which did not exceed five parasites per 100 microscopic fields. Twenty-two serial passages by blood transfer were made in chicks. There was a gradual rise in degree of parasitemia for six passages until approximately five or six parasites were present per microscopic field. At the end of the 11th passage, there were approximately 65 parasites per microscopic field, and this general level of parasitemia was maintained through the rest of the series. Beginning at about the 5th

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passage, the chicks began dying of the infection, and the mortality was about 55 per cent thenceforth.

After 22 passages in chicks, intravenous inoculations were made into domestic pigeons. Using inoculations of 3–6 ml. of infected blood, the parasitemia was brought from 0.2 to 76 parasites per field in five blood transfers, and the pigeons began dying on the 5th to 7th day of parasitemia.

Identification. As soon as mature gametocytes appeared in the isolated strain, their very close similarity to those of *Haemoproteus* was noted, and it was then realized that the parasites observed in the guinea fowl from which the isolation was made were gametocytes of *Plasmodium* rather than of *Haemoproteus*. Comparison of the morphology of the erythrocytic stages of the isolated strain with Schwetz's (1930) description of *P. fallax* indicated identity of the two parasites.

Schwetz described *P. fallax* from smears taken from an owl *Syrnium nuchale*. Since he was unable to find any additional owls with similar parasites, he was at a loss to decide whether he was dealing with a simple infection of a *Plasmodium* having gametocytes indistinguishable from *Haemoproteus* or with a mixed infection of *Plasmodium* and *Haemoproteus*. In one other owl of the same species he found gametocytes of what he considered to be *Haemoproteus*. He was unable to subject the question to the crucial test of transfer by blood inoculation. This we have been able to do and can now answer the question he raised very definitely. *P. fallax* has gametocytes indistinguishable from those of certain species of *Haemoproteus*. The infection which he described as *P. fallax* was very probably a single infection and the infection in the other owl which he took to be *Haemoproteus* was very likely an infection with *P. fallax* which, just as the one in our guinea fowl, was exhibiting predominantly gametocytes in its blood.

Description. The following description is based upon *Giemsa*-stained smears of infected pigeon's blood:

Uninucleate trophozoites. The youngest trophozoites are irregularly rounded, diamond-shaped, oval, or broadly spindle-shaped. "Ring" stages are present, but infrequent. Pigment granules, diffuse or definite and spherical. Pseudopod formation not prominent.

Schizonts. Approximately at the quadrinucleate stage elongation of the schizont begins and continues until it reaches from one pole of the host cell to the other. The schizont does not enlarge the host cell nor displace its nucleus. Pigment, an amorphous mass with occasional granules visible. When the schizont has attained the length of the nucleus of the host cell, sharply outlined, circular vacuoles appear and persist until the completion of schizogony. These vacuoles give an appearance of having been "punched out". Completed schizogony occurs when the form of the schizont is still elongated. Occasional segmenters have their merozoites distributed through the cytoplasm of the host cell. The mean numbers of merozoites per segmenter in four smears from four different pigeons were: 15.06 ± 0.23; 14.19 ± 1.01; 14.5 ± 0.36; 14.37 ± 0.38. The merozoites are round, usually with the chromatin peripherally arranged in a thick ring which leaves only a small central area of lighter color; no blue cytoplasm is visible in most of the merozoites. The compact, round, red appearance of the merozoites in the segmenter is similar to that of *P. hexamerium* and *P. rouxi*.

Gametocytes. These are typical "halteridium" shaped, indistinguishable from those of some species of *Haemoproteus.* They are broadly elongate, reach from pole to pole of the host cell and produce little or no displacement of the nucleus of the latter. The ends are rounded, or exceptionally pointed. The pigment is heavy, brown, spherical or slightly cylindrical. The "punched out" vacuoles are numerous. The two sexes exhibit the differences in staining common to other species of *Plasmodium* (or to *Haemoproteus*).

Mosquito hosts. Table 1 lists the dissection results of seven species of mosquitoes; three of them were fed upon infected chickens and six of them upon infected pigeons. *Aedes albopictus* was found to be the best vector of the species tested and over 60 experimental transmissions have been made with it. Susceptibilities of this mosquito are being tested to *fallax* infections in other avian hosts and will be subsequently reported elsewhere.

Pre-erythrocytic stages. The methods previously described by Huff and Coulston (1944) for studying pre-erythrocytic stages of *P. gallinaceum* in chicks were also employed here. Sporozoites were obtained from the salivary glands of infected

TABLE 1

Susceptibility of various mosquitoes to P. fallax *in chickens & pigeons*

SPECIES	FED ON CHICKENS			FED ON PIGEONS		
	Number fed	Number infected	Per cent infected	Number fed	Number infected	Per cent infected
Aedes triseriatus Say	—	—	—	20	5	25.0
Aedes atropalpus Coquillet	—	—	—	7	4	57.0
Aedes albopictus Skuse	—	—	—	261	191	73.0
Aedes aegypti Linnaeus	110	1	0.9	45	0	0
Anopheles quadrimaculatus Say	51	4	7.8	8	0	0
Culex pipiens Linnaeus	72	0	0	—	—	—
Culex quinquefasciatus Say	—	—	—	12	1	8.3

Aedes albopictus and either injected into the wing skin or intravenously. At various time intervals the birds were biopsied or killed and the tissues fixed. The data relative to the animals used in the study of pre-erythrocytic stages are given in table 2. Pre-erythrocytic stages were observed in areas of skin biopsied 24, 30, 40, and 42 hours after inoculation. They were observed in the organs of three birds inoculated intravenously, one killed after 48, one after 72, and the other after 96 hours. In the skin area taken 24 hours after inoculation a few small, uninucleate cryptozoites were observed in lymphoid cells and macrophages. Schizonts were observed in the 30-hour preparation. A fair number of the latter appeared to be in the fat cells of the skin. The parasites from either of these preparations would be difficult or impossible to distinguish morphologically from corresponding stages of *P. gallinaceum* in chickens. Schizonts, both pre-segmenters and segmenters, were found in the 40- and 42-hour preparations of skin. They resembled the corresponding stages of *P. gallinaceum* more than those of any other species yet studied and differed only in the smaller number of merozoites in the segmenters. The number in *P. gallinaceum* is approximately 70 to 100 whereas in *P. fallax* only about half that number was

found. Segmenters were observed in which all of the merozoites were peripherally arranged leaving a large central vacuole similar to the ones seen in *P. gallinaceum* (Huff & Coulston, 1944).

Pre-erythrocytic stages were observed only in the liver and spleen of birds inoculated intravenously with sporozoites. A single early schizont was found in the spleen of each of the birds killed 48 and 72 hours after inoculation. No pre-erythrocytic stages were found in any of the other principal organs. All of the parasites observed in the tissues of the bird killed 96 hours after inoculation appeared to have undergone some degeneration. One such abnormal schizont was observed in a Kupffer

TABLE 2

Data on pigeons used for the study of pre-erythrocytic stages of P. fallax.

TIME BETWEEN INOCULATION & FIXATION	NO. OF INFECTED MOSQUITOES USED	MANNER OF INOCULATION	PRE-ERYTHROCYTIC STAGES	ERYTHROCYTIC STAGES		
				Present (+) or absent (−)	Prepatent period (days)	Parasitemia at peak
18 hrs.	26	I. D.	−	−		
24 "	16	I. D.	+	−		
24 "	64	I. D.	−	−		
30 "	44	I. D.	+	+	9	50 per field
40 "	56	I. D.	+	+	7	4 "
42 "	24	I. D.	−	−		
42 "	20	I. D.	+	−		
48 "	25	I. D.	−	−		
48 "	51	I. D.	−	−		
71 "	49	I. D.	−	+	11	1 "
72 "	22	I. D.	−	+	8	8 "
93 "	54	I. D.	−	+	11	0.5 "
23 "	141	I. V.	−	(Killed during prepatent period)		
48 "	117	I. V.*	+	"	"	"
72 "	47	I. V.	−	"	"	"
72 "	145	I. V.*	+	"	"	"
96 "	179	I. V.*	+	+	4	1
8 days	80	I. V.	−	+	7	

* Squab; one week old or less
I. D. = intradermal
I. V. = intravenous

cell of the liver and several were found in macrophages of the spleen. It is, perhaps, significant that the only pre-erythrocytic stages found in the intravenous series were in young squabs (one week old or less).

As shown in table 2 all four possible combinations of pre-erythrocytic and erythrocytic stages were represented, namely, (1) neither were found, (2) both were found, (3) the pre-erythrocytic stages were found, but there was no parasitemia, and (4) there was parasitemia, but no pre-erythrocytic stages were found.

Phanerozoites. Search for exoerythrocytic stages in blood-induced infections and in the later stages of sporozoite-induced infections met with failure. Examination was made of the sectioned organs and brain smears of many heavily infected animals.

These negative findings are in contrast with the abundance of phanerozoites in infections with *P. gallinaceum*, *P. cathemerium*, and *P. relictum*.

Parasitemia. The results of infections of *P. fallax* in pigeons only will be presented here. Various other avian hosts are being used to test the infectiousness of the parasite for the host following (a) blood inoculations and (b) sporozoite inoculations, (c) to test the ability of the parasite to produce pre-erythrocytic stages in the host and

TABLE 3

Results of testing various breeds of pigeons with the sporozoites of Plasmodium fallax; *incidence in successive tests*

BREED OF PIGEON	FIRST TEST		SECOND TEST†		THIRD TEST‡	
	No. +	No. −	No. +	No. −	No. +	No. −
Barb	2	0				
Carneaux	8	3	0	2	2*	0
Flight, white	4	4	1	2	0	1
Frillback	1	2	0	1		
Hungarian, blue	2	1	0	1		
Jacobin	1	0				
Lahore	2	0				
Magpie	3	1	0	1		
Maltese	0	1	0	1		
Modena	5	5	3	2	0	1
Nun	4	0				
Pouter, Pigmy	3	1	1	0		
Roller, Birmingham	1	0				
Satinette	2	1				
Scandaroon	1	0				
Strasser	0	3	1	0		
Trumpeter, Russian	3	3	0	2		
Turbit	1	0				
Hybrids (F_1)	9	5	0	1		
Totals	52	30	6	13	2*	2
Percentage infected	63.4		31.5			

* 48 mosquitoes used.

† Performed only on birds which developed no infection during first test.

‡ Performed only on birds which developed no infection during first two tests.

(d) the infectiousness of gametocytes produced in the host for mosquitoes. These tests are in progress and will be reported later.

Eighteen pure breeds of pigeons and eight different kinds of F_1 hybrids between pure breeds were tested. The latter consisted of Silver King × Red Carneau, Lahore × Satinette, Lahore × Turbit, Modena × Ice, Pigmy Pouter × Barb, Scandaroon × Magpie, Scandaroon × Satinette, and Russian Trumpeter × Ice. A total of 82 individual birds was tested once. The birds which became infected from the first test were not tested again. Nineteen of the birds which developed no visible parasitemia from the first test were given a second test, and four of the birds which devel-

oped no parasitemia from either of the previous two tests were given a third test. Table 3 summarizes the incidence of parasitemia in the various breeds, and table 4 summarizes these experiments according to the degree of parasitemia. It is interesting to note that 84 per cent of the infections fall in the combined categories of 0, 1–100, 101–500 parasites per 10,000 red cells and that 11.1 per cent underwent

TABLE 4

Parasitemia in various breeds of pigeons bitten by mosquitoes infected with Plasmodium fallax

BREEDS	PARASITES PER 10,000 ERYTHROCYTES†						
	0	1–100	101–500	501–1000	1001–2500	2501–3000	over 3,000
Barb		1	1				
Carneaux	3	1	5		1		1
Flight	4	1	2				1
Frillback	2		1				
Hungarian	1	2					
Jacobin		1					
Lahore		2					
Magpie	1		2		1		
Maltese	1						
Modena	5	3				1	1
Nun		2				1	1
Pouter	1			1		1	1
Roller		1					
Satinette	1	2					
Scandaroon		1					
Strasser	3						
Trumpeter	3		2	1			
Turbit							1
Hybrids (F_1)	5	5	4				
Totals	30	22	17	2	2	3	6
Percentage of total	36.5	26.8	20.7	2.5	2.4	3.7	7.4
Second Test (Made only on birds negative to first test)							
11 breeds	13	1	3		1		
Percentage of Total	72.2	5.6	16.7		5.5		
Third Test (Made only on birds negative to first 2 tests)							
3 breeds	2	2*					

* Tested with 48 infected mosquitoes.

† Measured at peak of parasitemia.

parasitemias of 2500 or more per 10,000 red cells leaving only 4.9 per cent distributed over the intermediate grades (501 to 2500 parasites per 10,000 red cells). In the second test which, it must be remembered, was made upon birds which had shown no visible parasitemia from the first test, the percentage of birds with no parasitemia had increased to 72.2 as contrasted with 36.5 per cent from the first test. Similarly, the percentage of birds in the three lower levels of parasitemia (including the negative)

from the second test was 94.5 as compared with 84 per cent from the first test. The numbers from the third feeding are too small for comparison with the previous two tests. The two out of the four birds in which parasitemia was demonstrated received challenging doses of sporozoites from 48 infected mosquitoes each. The parasitemias resulting from these inoculations were 26 and 100 parasites per 10,000 red cells, respectively. It appears likely that with the standard infecting doses (bites from 10 infected mosquitoes), instead of the one given, these two birds would not have developed visible parasitemia.

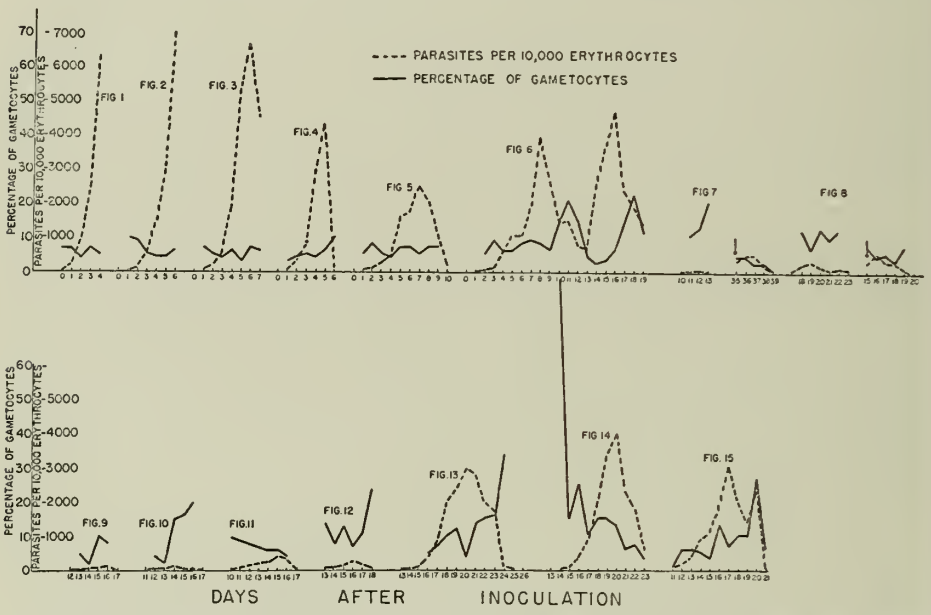
Course of infection. As indicated above and in table 4, infections in pigeons resulting from the bites of approximately 10 infected mosquitoes fall roughly into two categories on basis of peak parasitemia reached. Examples of low-grade infections are shown in figures 9 through 12 and examples of the highest parasitemias resulting from sporozoites are shown in figures 13 through 15. The duration of the parasitemia in the low-grade infections was five to seven days as contrasted with 10 to 12 days in the higher parasitemias. The character of the infection was similar to that of several other avian malarial infections. The percentage of gametocytes show interesting variations. The infection graphically shown in figure 14 was the result of the inoculation of sporozoites from 44 infected mosquitoes. The percentage of gametocytes was high on the first day of visible parasitemia, but followed a rapid, downward trend throughout the infection. Another type is illustrated in figure 15. This infection resulted from the bites of 10 infected mosquitoes. In this instance, the percentage of gametocytes was very low on the first day of visible parasitemia, but pursued an upward trend until the final day of parasitemia. The possible significance of these differences will be discussed below.

In all, 58 infections resulting from sporozoites were studied. The average number of infected mosquitoes used was 12.5 $\pm$ 1.14, the average prepatent period was 9.2 $\pm$ 1.7 days, and the average number of parasites per 10,000 red cells (at the peak of parasitemia) was 1003 $\pm$ 13. Calculations of the coefficients of correlation (a) between number of mosquitoes used and prepatent periods, (b) between number of mosquitoes used and number of parasites per 10,000 red cells, and (c) between prepatent periods and number of parasites per 10,000 red cells indicated no, or questionable, correlation in all three instances.

There was also considerable variation in the types of infection resulting from the transfer of infected blood to uninfected pigeons. Figures 1 and 2 represent the type for a fairly large percentage of infections resulting from the inoculation of a sufficient number of parasites to produce parasitemia in the recipient bird within 24 hours after inoculation. Such infections rose rapidly to 6000 or 7000 parasites per 10,000 red cells and resulted in the death of the host on the 5th or 6th day. Other birds with the same size of inoculation lived past the crisis (figures 2 and 3), but in some instances died within a few days after the crisis. Others (figure 4) underwent a parasitemia more like that in sporozoite-induced infections in that the increase in numbers of parasites was slower and the peak of parasitemia was lower than in the infections illustrated by figures 1–4. Figure 6 represents an infection similar to the last mentioned category except that this bird underwent a relapse immediately following the lowering of the parasite level from the initial infection. It is interesting to note that

the highest percentage of gametocytes attained in the initial infection was approximately equal to the highest percentage in the relapse.

Two birds were given inoculations of sporozoites followed by large inoculums of infected blood. The first (Figure 7) received the sporozoites from 22 mosquitoes intradermally, had a prepatent period of nine days, and underwent a slight parasitemia. After 22 days 4 ml. of infected blood was given intravenously which produced a parasitemia of 250 parasites per 10,000 red cells immediately following the inoculation. The second (figure 8) received the sporozoites from 56 mosquitoes intrader



Explanation of figures: Figures 1 to 15 all show graphically the course of infection of *P. fallax* in individual domestic pigeons. Parasite counts per 10,000 red cells are shown by the broken line and the percentage of gametocytes by the solid line. Figures 1–6 represent infections which were induced by intravenous inoculations of erythrocytic parasites. Figures 7 and 8 represent sporozoite-induced infections followed by infections produced by subsequent inoculations of erythrocytic parasites (see arrows). Figures 9–15 represent infections induced by sporozoites. Figure 14 depicts an infection resulting from the intradermal inoculation of sporozoites from 44 infected mosquitoes. Figure 10 depicts an infection resulting from the bites of 8 infected mosquitoes and figures 9, 11, 12, 13, and 15 depict infections resulting from the bites of 10 infected mosquitoes each.

mally, had a prepatent period of 10 days and also underwent a slight parasitemia. Eighty days after the disappearance of parasites from the blood 6 ml. of infected blood was given intravenously which gave immediately a parasitemia of 230 parasites per 10,000 erythrocytes. It is interesting to note that the percentage of gametocytes is significantly higher in each bird in the infections resulting from sporozoites than in the second infection which was the result of inoculating infection blood. In the converse experiment in which the inoculation of blood preceded the inoculation of sporozoites, no visible parasitemia resulted from the latter inoculation.

Periodicity. Pigeons inoculated with infected blood were kept on a 12-hour light, 12-hour dark schedule during the entire course of infection, and blood smears were made at 4-hour intervals. Counts were made from the smears of the numbers of uninucleate stages, of 2–5 nucleate stages, of stages with more than five nuclei, of segmenters, and of gametocytes. There was no evidence of synchronism of schizogony in these counts.

DISCUSSION

Discovery of a *Plasmodium*, the gametocytes of which are indistinguishable from those of *Haemoproteus* introduces a complexity into the diagnosis of blood infections in wild birds and renders suspect many of the records of *Haemoproteus* infections already in the literature. If, as in the case of the guinea fowl from which we isolated *P. fallax*, infections of this species tend to be chronic and to have mostly gametocytes in the blood there is no known criterion for distinguishing them from infections of *Haemoproteus* when only a single blood smear is available. Since Schwetz found *P. fallax* in an owl, and we have observed or produced infections in guinea fowl, chicks, pigeons, and canaries, it would appear that its host specificity is not very strict. There would appear then to be a very good chance to isolate it in laboratory birds if opportunity is presented for obtaining blood from the infected bird. The deceptive nature of this species is indicated by the name *fallax* given to it by Schwetz. The Latin *fallax* means deceptive or confusing.

A word of caution is, perhaps, needed in connection with the recording of susceptibility of any species of mosquito to a particular parasite. Infection or lack of infection of a mosquito is determined by many factors including (a) those inherent in the individual mosquito, (b) the type of host in which the gametocytes are produced, and (c) the particular stage in the infection at which the mosquito has taken its infectious meal. A table of dissection results such as table 1 should not be tacitly interpreted as representing the inherent susceptibility of mosquitoes unless some attention is given to controlling the other factors which take part in aiding or preventing infection. The gametocytes of the *1P* strain of *P. relictum* are not infectious for mosquitoes when produced in pigeons, but are very infectious for several species when produced in canaries. Lumsden and Bertram (1940) and Cantrell and Jordan (1946) have shown different degrees of infectivity of gametocytes of *P. gallinaceum* at different stages in the course of the infection in chickens. Although it is possible that the factors which take part in determining whether a mosquito will be immune to infection may be different from those which determine the degree of infection, it appears likely that incidence of infection would be influenced by the latter through shifting certain individuals from the infected to the uninfected columns or *vice versa*.

At the present stage in our knowledge of pre-erythrocytic stages of malaria, it is important to begin to reach some kind of generalizations about their behavior. It is interesting in this regard that the pre-erythrocytic stages of *P. fallax* fall into the pattern of behavior known from four other species of avian malaria (*P. gallinaceum*, *P. cathemerium*, *P. relictum*, and *P. lophurae*). Morphologically, they are all fairly similar. They appear not to differ widely in the types of host cell preferred nor in the length of time required for the growth of one generation. It appears to be signifi-

cant that the first five avian species for which the pre-erythrocytic stages have been studied have been so consistently alike. As the pre-erythrocytic stages of each new species to be studied is found to fall into this pattern, the likelihood that the pattern is general for all avian species of malaria becomes more probable.

Pre-erythrocytic stages of *P. fallax* are not as abundant in pigeons as the corresponding stages of *P. gallinaceum* in chickens, or *P. cathemerium* or *P. relictum* in canaries. Whether this is a real difference in the species of parasites or possibly a difference in their behavior in different hosts is not known at present. It is also true that phanerozoites of *P. fallax* were not found in pigeons, whereas the corresponding stages of the above species are prevalent in blood-induced infections in their respective hosts. In these respects, *P. fallax* behaves more like *P. vivax* than do other avian species of malaria. Whether or not they have chemotherapeutic similarities is a subject under investigation.

It has already been mentioned that no correlation could be found between numbers of infected mosquitoes used and the height of the resulting parasitemia. Although the majority of the infections were produced from the bites of 10 infected mosquitoes, several were produced by numbers of mosquitoes ranging between 16 and 80. The majority of sporozoite-induced infections—whether resulting from a small or large number of infected mosquitoes—do not attain a parasitemia of more than 500 parasites per 10,000 red cells. An appreciable number (11 per cent, however, attain parasitemias of over 2500 per 10,000 red cells, even though they received approximately the same inoculum as the group with lower parasitemias. When birds which did not show any visible parasitemia were subjected to a second test approximately twice as many failed to develop parasitemia as in the unselected birds of the first test. Two possible explanations are suggested for this difference. (1) The sporozoites from the first inoculation may have produced some immunity either from the antigen in the sporozoites themselves or from possible resulting inapparent infections. (2) Individual pigeons may possess different degrees of innate immunity to the parasite and, irrespective of any acquired immunity from previous inoculations or variations in the general state of their health, they may because of their genetic constitution react in similar manner when inoculated a second or third time. The fact that sporozoite-induced infections fall into two categories regardless of the size of the inoculum appears to favor the second explanation above. However, it is possible that both explanations could partially apply.

The types of infection produced by inoculation of infected blood differ in several respects from those produced by sporozoites. The majority of blood-induced infections are characterized by higher parasitemia and (in non-fatal infections) by the earlier production of a crisis. Intravenous inoculation of large numbers of erythrocytic parasites eliminates the prepatent period and allows the parasites to attain an ascendancy over the immune mechanisms of the host before the latter have been effectively mobilized. Since this mobilization occurs in three to five days after inoculation, it is not surprising that many of the sporozoite-induced infections are characterized by low parasitemia, since none of the erythrocytic stages appear in the blood stream before eight or ten days. A possible explanation of the occasional similarity between a blood-induced and a sporozoite-induced infection (as illustrated

in figures 5 and 13) may be in the natural immunity possessed by the two pigeons. The blood-induced infection represented by figure 5 may have been delayed and diminished by a higher than usual natural immunity, whereas the sporozoite-induced infection illustrated by figure 13 may have met less than usual in the way of natural immunity. According to this view, immunity—both natural and acquired—plays a more important part than the kind of the infecting stage, in determining the type and degree of parasitemia.

In general, the percentages of gametocytes are higher in sporozoite- than in blood-induced infections. This is shown not only in the higher average percentage of gametocytes in sporozoite-induced infections, but is strikingly revealed by the infections resulting from double inoculations illustrated in figures 7 and 8. In each instance, the percentage of gametocytes in the initial sporozoite-induced infection is significantly higher than in the subsequent blood-induced super-infection. Evidence presented by other authors has indicated a close relationship between exoerythrocytic stages and gametocytes. This would be the logical way to explain the larger proportion of gametocytes in sporozoite-induced infections. Adler and Tchernomoretz (1943) found that, in chicks infected with *P. gallinaceum* and treated with quinine, discontinuance of the therapy after small erythrocytic parasites had appeared was followed by parasitemia with mature gametocytes. Lewert (1950) found a higher percentage of gametocytes in infections of chickens induced by the inoculation of exoerythrocytic stages of *P. gallinaceum* from a routine brain passage strain than he found in infections induced by inoculations of erythrocytic parasites. If we assume that a certain proportion of erythrocytic merozoites regularly go into the production of gametocytes and that exoerythrocytic merozoites have the ability to produce gametocytes without an intermediate schizogony the increased percentages of gametocytes in infections induced by either sporozoites or exoerythrocytic stages would be explained by the addition of the gametocytes produced from both sources. It is particularly interesting in this connection to examine figure 14 which represents the parasitemia resulting from the intradermal inoculation of the sporozoites from 44 heavily infected *Aedes albopictus*. The percentage of gametocytes on the first day of visible parasitemia was 86 and declined with minor fluctuations to three on the last day smears were taken. This preponderance of gametocytes early in the infection may possibly be the result of the unusually large number of sporozoites—and, therefore, of pre-erythrocytic stages—involved. Since it was shown by Huff and Gambrell (1934) that asexual parasites are removed from the blood of an immune bird more rapidly than the gametocytes, it is not surprising to observe a rapid increase in the percentage of gametocytes after the crisis in the infection. This is shown in figure 6 (blood-induced) and in figures 12, 13, and 15 (sporozoite-induced). This terminal rise in percentage of gametocytes is a result of the differential rate of removal by the immune mechanisms of the host. Therefore, any large percentage of gametocytes early in the infection when the immune mechanisms are ineffective must have some explanation such as the suggestion that exoerythrocytic merozoites may produce gametocytes directly.

If a certain percentage of merozoites of a given strain of malaria, regardless of whether they are of erythrocytic or exoerythrocytic origin, are destined to produce

gametocytes, it would follow that the genetic makeup of such merozoites requires the erythrocyte as an environment for its expression. If these hypotheses be correct, it then becomes interesting to speculate upon what happens to an exoerythrocytic merozoite having the potentialities for producing a gametocyte when it enters some cell other than an erythrocyte.

SUMMARY

1. Laboratory studies were made on a strain of *Plasmodium fallax* Schwetz isolated from an Uganda tufted guinea fowl, *Numida meleagris major* Hartlaub taken in the Anglo-Egyptian Sudan.

2. This strain produced light infections in chicks, but was more intensively studied in domestic pigeons in which it produced high parasitemias and a high mortality.

3. Experimental infections were obtained in *Aedes triseriatus*, *A. atropalpus*, *A. albopictus*, *A. aegypti*, *Anopheles quadrimaculatus*, and *Culex quinquefasciatus*, but not in *Culex pipiens*. *Aedes albopictus* was the best vector.

4. Pre-erythrocytic stages were observed in injection sites of the skin biopsied 24, 30, 40, and 42 hours after inoculation and in the liver and spleen of three pigeons intravenously inoculated with sporozoites and killed 48, 72, and 96 hours after inoculation. Morphologically, the pre-erythrocytic stages resembled the corresponding stages of *P. gallinaceum* very closely. No phanerozoites were found.

5. A total of 82 pigeons belonging to 18 different breeds and eight different first crosses were bitten by an average of 12.5 infected mosquitoes. Fifty-two (63.4 per cent) of these developed parasitemias following the first test. Nineteen of the pigeons which were refractory to the first test were given a second test; six (31.5 per cent) of these became infected. Four of the 13 which were refractory to two previous tests were tested a third time; two of them (which received the sporozoites from 48 mosquitoes) developed parasitemia.

6. Eighty-four per cent of the birds receiving sporozoite inoculations underwent low infections (not more than 500 parasites per 10,000 red cells) or were entirely refractory. About 11 per cent underwent parasitemias of 2,500 or more parasites per 10,000 red cells.

7. Considerable variation was found in infections induced in pigeons by inoculation of erythrocytic stages. Some infections attained a parasitemia of 6,000 to 7,000 parasites per 10,000 cells in three to five days after inoculation and terminated fatally. Others attained as high a parasitemia, but underwent a crisis before death of the bird and in still others the crisis was followed by recovery of the host.

8. The percentage of gametocytes in sporozoite-induced infections was higher than in blood-induced infections.

9. No synchronism was observed in the schizogonic reproduction in the blood.

10. Proof of the existence of a species of *Plasmodium* the gametocytes of which are identical in appearance to the gametocytes *of Haemoproteus* emphasizes the need for caution in identifying the parasites in blood smears from wild birds, when gametocytes only are found.

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STRAIN DIFFERENCES IN *PLASMODIUM GALLINACEUM* BRUMPT

I. Differences in the Behavior of the Exoerythrocytic Forms of a Blood-passaged (BI) and Sporozoite-passaged (SP) Strain of *Plasmodium gallinaceum*

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Haas *et al.* (1946 and 1948) were able to produce exclusively exoerythrocytic (EE) infections of *Plasmodium gallinaceum* in young chicks by inoculating brain tissues of birds dying of exoerythrocytic infections. In the same year Lewert (1948 and also 1950 and 1950A) produced infections of this type by inoculating exoerythrocytic parasites of *P. gallinaceum* grown in tissue cultures. Haas *et al.* (1948) further reported that the sporozoites of a strain transferred consecutively by the direct inoculation of infected blood produced exclusively exoerythrocytic infections.

The work reported in this paper deals with two different strains of *P. gallinaceum* derived from the same source, the 8A strain, but differing in that one strain was passaged exclusively by blood transfer and the other exclusively by sporozoites. The inoculation of exoerythrocytic parasites derived from one strain (BI) resulted in infections showing exclusively exoerythrocytic parasites while the inoculation of these same forms from the other strain (SP) produced infections exhibiting both exoerythrocytic and erythrocytic parasites.

MATERIALS AND METHODS

A single strain of week-old New Hampshire Red chicks weighing 45 to 55 grams was used in all experiments. Two strains of *P. gallinaceum*, both derived from a common source, were used. At the beginning of these experiments one strain (BI) had undergone 152 consecutive weekly blood transfers. The other strain (SP) had been passaged exclusively through the mosquito approximately 55 consecutive times.

Prior to these studies the behavior of neither of these strains could be considered unique. When the BI strain was transferred by the inoculation of infected blood the resultant infections always produced a diphasic death curve, first described by James (1939) wherein most of the chicks died during the first two weeks after inoculation of causes attributable to acute parasitemia. Those which survived this phase of the infection usually died 16 to 24 days after inoculation of causes attributable to exoerythrocytic (phanerozoite) parasites. The sporozoite-induced infections of the SP strain, as described by Coatney *et al.* (1945), are characterized by the development of a subacute parasitemia which develops in the second week after inoculation, accompanied by an overwhelming exoerythrocytic infection, which causes the death of almost all the chicks within two weeks after inoculation.

Infected chicks, moribund at a period in their infection when exoerythrocytic parasites could be expected to be abundant, were used as donors. An estimate of the parasitemia in the donor was made immediately prior to sacrifice. Immediately after sacrifice, a compression smear of the brain cortex was made and examined for the presence and density of tissue forms of the parasite. The brain was then removed and ground to a fine suspension (90 seconds in a micro-Waring blendor) in 25 ml. of 0.85 per cent NaCl. The suspension was drawn into a small syringe through a 24 gauge needle. Experimental chicks received 0.1 ml. of the suspension intramuscularly in the pectoral region.

The experimental chicks were examined for parasitemia daily beginning on the 6th or 7th day after inoculation and continuing, in survivors, for at least two weeks. A chick was considered negative for parasitemia, if no parasites were observed in 30 microscopic fields of approximately 100 erythrocytes each. Compression smears were made from the brain of each chick that died, as well as those sacrificed on the 40th post inoculation day. These were examined for the presence and density of exoerythrocytic parasites. A brain compression smear was considered negative for exoerythrocytic parasites if none was found during a three to five minute examination of the smear. If one exoerythrocytic form was found in such a smear, the infection was considered +; if less than half the capillaries had such forms, ++; if more than half, but not all had such forms, +++; and if all the capillaries examined had exoerythrocytic forms, ++++.

The infections in the chicks fell into five well-defined patterns: (1) Acute, fulminating, fatal parasitemia with no exoerythrocytic parasites found in brain smears (ER infection); (2) Acute parasitemia with partial recovery followed by death in one to two weeks from an overwhelming exoerythrocytic infection (ER-EE), (Patterns (1) and (2) are characteristic of blood-induced BI strain infections); (3) Death from an overwhelming exoerythrocytic infection with no normal pigmented erythrocytic parasites detected (EE); (4) An apparent simultaneous development of acute parasitemia and exoerythrocytic infection, with death attributable to the latter (EE-ER), (Pattern (4) is characteristic of the sporozoite-induced SP strain infections); (5) Subacute parasitemia, followed by a chronic infection (er).

Details of the methods used for infecting chicks by intravenous inoculation of infected blood are given in Coatney and Sebrell (1946). The methods used for inoculating chicks with sporozoites were the same as those used by Coatney *et al.* (1945).

EXPERIMENTAL

Two parallel experiments were carried out using brain-tissue parasites to infect the recipients. The *donors* for the first experiment (table 1) were inoculated with trophozoites of the BI strain and the *donors* for the second experiment (table 2), with sporozoites of the SP strain. Five to ten recipients were inoculated at each transfer. In each series, five of the donors were treated with quinine sulfate, at a dosage of 0.2 mg./gm. twice daily throughout the course of the infection, in order to suppress the number of erythrocytic parasites transferred with the brain inoculum.

Of the 110 chicks inoculated with parasites of the BI strain 83 became infected (table 1). Of those infected, 69 per cent had EE infections; the remainder had a few normal erythrocytic parasites in their blood. In the cases where the donors were

quinine-treated, or had naturally low parasitemia at the time of sacrifice, the infections in the recipients were exclusively EE.

Of the 89 chicks inoculated with parasites of the SP strain, 73 developed infections (table 2). All of the latter had normal erythrocytic parasites in the circulating blood even when the donors were treated with quinine. Sixty-four per cent of the infected chicks had EE-ER infections, the remainder had infections of the er type.

In two additional series of experiments, each of the strains was passaged for ten consecutive times by the inoculation of brain tissue infected with exoerythrocytic

TABLE 1

Pattern of P. gallinaceum *infections resulting from the inoculation of brain tissue containing exoerythrocytic parasites of the BI strain*

DONOR CHICKS				RECIPIENT CHICKS					
Passage No.	Treatment	Parasitized rbc/10^4 rbc	Density of exoerythrocytic forms	No. inoculated	Patterns of infections				
					O	ER	EE	EE-ER	er
153	None	420	0	5	5	0	0	0	0
153	None	2850	+	5	3	2	0	0	0
153	None	2500	0	5	3	1	1	0	0
154	None	6700	++++	5	1*	0	2	2	0
154	None	290	++++	5	0	0	5	0	0
155	Quinine†	210	++++	5	1*	0	4	0	0
156	None	5000	++++	5	0	0	4	1	0
156	None	20	+++	5	1	0	3	0	1
157	None	2200	++++	5	0	0	3	1	1
157	None	170	++++	5	1	0	3	0	1
160	Quinine	40	++++	5	1*	0	4	0	0
160	Quinine	80	++++	5	1*	0	4	0	0
160	Quinine	20	+++	5	2	0	3	0	0
160	Quinine	20	++	5	2*	0	2	0	1
162	None	No data	+++	10	4	1	3	2	0
165	None	4100	+++	10	1*	0	4	5	0
168	None	2200	++++	10	0	0	3	6	1
170	None	80	+++	10	1	0	9	0	0
Totals				110	27	4	57	17	5

* Chicks died with no detectable infection.

† Quinine was administered at 0.2 mg./gm. twice daily throughout the course of the infection.

parasites. All of the recipients of the BI strain had EE infections while only two of those of the SP strain had this pattern of infection (table 3).

It is conceivable that the differences observed in the patterns of infections produced in the recipients of the two strains could have resulted from the method of passage of the infection. If this were the case, one would expect that blood-passage of the SP strain would alter the behavior of the exoerythrocytic parasites of this strain and conversely, mosquito-passage of the BI strain should yield exoerythrocytic parasites which would produce normal erythrocytic parasites when inoculated into susceptible recipients.

TABLE 2

Pattern of P. gallinaceum *infections resulting from the inoculation of brain tissue containing exoerythrocytic parasites of the SP strain*

DONOR CHICKS				RECIPIENT CHICKS					
Passage No.	Treatment	Parasitized rbc/10^4 rbc	Density of exoerythrocytic forms	No. inoculated	Patterns of infections				
					O	ER	EE	EE-ER	er
55	None	2300	+++	5	0	0	0	2	3
55	None	5800	++++	5	0	0	0	3	2
55	None	4800	+++	5	2	0	0	1	2
56	None	3150	+++	5	0	0	0	5	0
56	None	2750	+++	5	2*	0	0	3	0
56	None	2200	++++	5	1	0	0	2	2
56	None	2700	++++	5	0	0	0	2	3
57	None	2500	++++	5	0	0	0	5	0
58	Quinine†	3100	++++	5	1	0	0	3	1
58	Quinine	30	++	5	1	0	0	2	2
58	Quinine	630	+++	5	2	0	0	1	2
58	Quinine	3100	++++	5	1*	0	0	4	0
58	Quinine	40	++	5	2*	0	0	3	0
60	None	5800	+++	5	2	0	0	1	2
60	None	2800	+++	5	0	0	0	2	3
60	None	6100	+++	4	0	0	0	2	2
60	None	7100	++	5	1	0	0	2	2
60	None	20	++++	5	1	0	0	4	0
Totals				89	16	0	0	47	26

* Chicks died with no detectable infection.

† Quinine was administered at 0.2 mg./gm. twice daily throughout the course of the infection.

TABLE 3

Patterns of P. gallinaceum *infections produced in young chicks during ten consecutive brain-tissue passages of the BI and SP strains*

STRAIN	NUMBER OF CHICKS		PATTERNS OF INFECTIONS			
	inoculated	infected	ER	EE	EE-ER	er
BI	98	96	0	96	0	0
SP	92	91	0	2	72	17

TABLE 4

Patterns of P. gallinaceum *infections in young chicks inoculated with brain tissue containing exoerythrocytic parasites of the SP strain*

BLOOD PASSAGE NUMBER	DENSITY OF EE FORMS IN DONOR	NO. CHICKS INOCULATED	RESULTING INFECTIONS				
			O	ER	EE	EE-ER	er
1	++++	10	1	0	0	9	0
10	++++	20	0	0	0	16	4
16	+++	10	1*	0	0	9	0
25	++++	10	0	0	0	10	0
53	++++	10	0	0	0	10	0

* Died before erythrocytic parasites were detected.

In order to test the first possibility the SP strain was blood-passaged 53 consecutive times at weekly intervals. The resulting infections (ER or ER-EE) were superficially indistinguishable from those of the blood-passaged BI strain. However, when at the 1st, 10th, 16th, 25th, and 53rd blood passage, the infection was also transferred by the inoculation of infected brain tissue no EE infections resulted (table 4, cf. table 1). Furthermore, at the 26th and 53rd blood passage the infection was also passaged through *Aedes aegypti*; ten chicks were inoculated at each passage. All the twenty chicks exhibited typical EE-ER infections which could not be distinguished from those produced by the original SP strain, as described by Coatney *et al.* (1945).

The second possibility is disproved by the data of Haas *et al.* (1948), who showed that when the BI strain is mosquito-passaged the resulting infections were exclusively EE. Our experience parallels that of these investigators as will be shown in a subsequent paper.

DISCUSSION

Both strains of *P. gallinaceum* described in this paper originated from a common source but differed in their method of transfer and in the type of infection produced. The BI strain was passaged exclusively by transfer of infected blood, while the SP strain was passaged through mosquitoes. Both strains produced abundant phanerozoites (late exoerythrocytic parasites). However, the phanerozoites of the SP strain were able to produce merozoites which developed into normal, pigmented, multiplying trophozoites, but the phanerozoites of the BI strain were unable to do so. Consequently, the inoculation of the phanerozoite-infected brain tissue produced different patterns of infections depending on the strain used. The phanerozoites of the BI strain generally produced overwhelming exoerythrocytic (EE) infections, and in the few instances in which normal erythrocytic parasites were found their presence could be accounted for on the basis of erythrocytic parasites being present in the inoculum. Where the latter were absent or rare, as in quinine-treated chicks, or in chicks exhibiting low parasitemia, the infections produced in the recipients were exclusively exoerythrocytic. On the other hand, normal erythrocytic parasites always developed in infections produced by phanerozoites of the SP strain.

The loss of the ability of phanerozoites to produce merozoites which would reproduce normally in the erythrocyte also extended to the pre-erythrocytic form of the parasites, the metacryptozoites of Huff and Coulston (1944). Thus when the BI strain was passaged through the mosquito the resulting sporozoites produced an overwhelming exoerythrocytic infection with exceedingly rare normal erythrocytic trophozoites or gametocytes (cf. Haas *et al.*, 1948).

The SP strain retained its characteristic of EE and ER parasites through 53 consecutive blood transfers and therefore the differences noted in these two strains can hardly be attributed solely to their method of passage. It is possible that in the course of transfer of the BI strain a mutant or mutants of equal or greater survival value than the parent arose spontaneously. This mutant then retained the ability to invade the endothelium of the host but lost most of its ability to reinvade the erythrocyte. While the parent strain was being passed by the abnormal method of

blood transfer it could survive and produce infective gametocytes. However, in view of the fact that parasites which develop from sporozoites are exclusively fixed-tissue parasites and, as pointed out earlier, these were unable to produce merozoites which could invade the red blood cells, serial passage of this strain by mosquitoes would become almost impossible. The BI strain then became a completely aberrant strain.

The question occurs: If the BI strain were the result of a mutation, why had this mutation not occurred in the mosquito-passaged strain? The answer seems to lie in the fact that the mutation was relatively rare, since (1) it failed to occur in any of the 53 chicks which served as consecutive donors in the blood transfer of the SP strain and (2) even though the chances of a mutation occurring in the mosquito-passaged strain were as frequent as in a blood passaged strain, it would have had a lesser chance of being selected. First, donors for the SP strain were specifically chosen because they had gametocytes. This would automatically exclude any chicks that died before these appeared. Secondly, one bird-mosquito-bird cycle takes a total of three to four weeks, so that over the same period of time the BI strain underwent three to four times as many transfers as the SP strain. Thirdly, based on numbers of parasites successfully transferred in each passage by each method there would be many times the chance of a mutant being selected for further passage by the blood-transfer method than by the mosquito-passage method.

SUMMARY

Infections resulting from the inoculation of brain tissue infected with exoerythrocytic parasites of two strains of *Plasmodium gallinaceum*, originally from the same source, have been described. Under these conditions exoerythrocytic forms of:

1. The BI strain were unable to produce merozoites which could reproduce normally in the erythrocytes;
2. The SP strain were able to produce merozoites which could reproduce normally in the erythrocytes.

The characteristics of the exoerythrocytic parasites of these strains were not changed by the method of transfer of infection, whether by blood, infected brain tissue, or by sporozoites.

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STUDIES ON RELAPSES IN BLOOD-INDUCED INFECTIONS FROM *PLASMODIUM MALARIAE* AND *PLASMODIUM CYNOMOLGI*

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Discussions on the origin of relapses in haemosporidian infections began with the very first studies on the subject of malaria. Golgi (1893) expressed the opinion that the malignant tertian parasites phagocyted by leucocytes and macrophages may continue their development inside the cytoplasm of the phagocyting cells. This development, according to Golgi, could explain the origin of relapses. Marchiafava and Bignami (1902), on the basis of observations made by Bignami and Bastianelli did not support Golgi's hypothesis, and considered human malaria relapses as resulting from the multiplication of parasites of the endoerythrocytic cycle which had escaped from the action of drugs and from the defensive powers of the host.

Bignami (1910) returned to the subject with the statement that relapses should be considered in relation to the persistence of endoerythrocytic parasites: "In a group of relapses, the parasitic material which maintains the infection is represented by a minimal amount of forms of the endoerythrocytic cycle: these may be occasionally observed by an accurate microscopic examination at least in some moments during the latency, and their number and virulence may be for a long time not sufficient to produce fever. Chiefly in the cases in which groups of relapses are allowed to develop without the influence of the specific therapy, it is possible that after a number of attacks of fever, the human organism acquires some immunity against the pyrogenous action of the parasites. This immunity, being as in other infections transitory, may attenuate or end in a lapse of time, giving so origin to a relapse."

Bignami's observations and his explanation of the origin of relapses based on the immunological balance between the parasites and the host were probably too pioneer a work in 1910 to be universally accepted or even understood. The problem of relapses therefore continued to give rise to many theories. Among them it is sufficient to recall the theory of Schaudinn, who considered relapses as dependent on a schizogonic process of the macrogametocytes. This theory of Schaudinn was never confirmed and is no longer supported by anyone.

After the existence of an endohistiocytic cycle was observed in some species of haemosporidia, the claim was made that forms of the endohistiocytic cycle, persisting during the various periods of latency of the infection, could be responsible for relapses. This theory was intended to give a general explanation of relapses in all haemosporidian infections, including those of human malaria.

Such a theory did not pay sufficient attention to the fact that the endohistiocytic cycle is observable only in some species of bird and reptilian haemosporidia, having never been demonstrated in mammalian haemosporidia. Moreover, researches made

by one of us (Corradetti 1940, 1941) led to the conclusion that relapses, even in a species with an endohistiocytic cycle, as *Plasmodium gallinaceum*, are dependent on endoerythrocytic forms. This is demonstrated by the fact that the endohistiocytic cycle of *P. gallinaceum* disappears completely in the second month after inoculation, while endoerythrocytic parasites in the blood and recent pigment in the phagocytes of the internal organs have been observed for 48 weeks after the beginning of the infection.

After the discovery by Shortt, Garnham and Malamos (1948) of the existence of an hepatic endoepithelial cycle of *P. cynomolgi* in monkeys, followed soon after by similar results with *Plasmodium vivax* and *Plasmodium falciparum* in man, the hypothesis was suggested that in these species relapses could have their origin in this cycle.

This hypothesis could find a basis in the observation by Shortt and Garnham (1948) of hepatic endoepithelial forms in one monkey three months after the inoculation of sporozoites. These authors, while recognizing that the continuance of an hepatic endoepithelial cycle is not clear scientific proof that this is the source of clinical relapses, show a tendency to accept this conclusion. Their opinion on the subject is expressed in the following terms: "The inoculation of sporozoites by the infected mosquito is followed by a pre-erythrocytic development in the parenchyma cells of the liver, with the ultimate production of merozoites. Many of these enter the erythrocytes to produce a parasitaemia and a clinical attack of malaria. Other merozoites enter normal liver cells and repeat the process of exoerythrocytic schizogony. This latter process repeats itself indefinitely, irrespective of whether the erythrocytic is present or is in abeyance as the result of antimalarial treatment or a naturally acquired active immunity. This active immunity is operative only against the erythrocytic parasites and destroys those merozoites liberated by the exoerythrocytic schizonts which are destined to enter red cells. Those which enter liver cells to maintain the exoerythrocytic cycle are protected from this immunity by their intracellular position outside the circulating blood. If, for any reason, the active immunity of the host is impaired it no longer operates against the merozoites destined to start the erythrocytic cycle, and these enter the red cells and initiate a clinical relapse."

Another outstanding argument of those who support the endohistiocytic, or the endoepithelial origin of relapses, is the widespread opinion that in sporozoite-induced infections frequent relapses would take place, which would be almost entirely absent in blood-induced infections. It is claimed that while in sporozoite-induced infections, relapses may arise from a persisting endohistiocytic cycle (or endoepithelial in monkeys and man), this is not the case in blood-induced infections, in which only endoerythrocytic forms are artificially introduced in the host's body.

The main object of the researches involved in the present paper has been to establish whether in mammalian malaria a number of serial passages through the vertebrate host, all obtained through blood inoculation, would ultimately lead mammalian plasmodia to produce infections showing a tendency to end after the primary attack, without giving rise to relapses. This would have to be expected if it is assumed to be true that relapses derive from endoepithelial forms only, and not from endoerythrocytic parasites: the latter being introduced into the host body only by blood inoculation, no relapses could occur if the theory is correct.

The behavior of relapses in serially-induced infections by means of blood inoculation has been studied on two plasmodia: *P. malariae* of man and *P. cynomolgi* of monkeys.

OBSERVATIONS ON *PLASMODIUM MALARIAE*

The researches were made on patients inoculated in serial passages by means of *P. malariae*-infected blood. The original infected blood from which we started the series of passages was given us by another institute and was derived from an indefinite number of inter-human passages.

Thirty-five passages from man to man were made in our experiments. Our attention was centered on eight subjects, pertaining respectively to the 2nd, 15th, 17th, 19th, 20th, 23rd, 31st, 34th passages of the series. All these subjects were carefully studied for a period of 35 weeks from the beginning of the infection. Their blood was examined daily during the entire primary attack until the disappearance of the parasites (spontaneous or provoked by quinine); the blood was subsequently reexamined each time an elevation of temperature was observed. All the patients showed more or less persistent parasitic relapses at a time varying from two to 29 weeks after the end of the primary attack. In two subjects in particular, it was observed that *P. malariae*, after 31 and 34 inter-human passages respectively (all made through blood inoculation), still maintained the property of producing relapses 22 and 29 weeks after the end of the primary attack.

OBSERVATIONS ON *PLASMODIUM CYNOMOLGI*

The researches on *P. cynomolgi* were made on 21 monkeys of the species *Macacus rhesus:* they were all inoculated with infected blood in 12 serial passages. Seven of these monkeys belong to other experiments made by Dr. G. Gramiccia of the same institute, and we thank him for use of his very helpful data. As these seven monkeys had been studied for another purpose, there was some discontinuity in their blood examinations. All the other monkeys were observed daily by blood examination, from the beginning to the end of the experiment.

Observation of the 21 monkeys continued for a period varying from six to 39 weeks after inoculation. All the infections were allowed to follow a spontaneous course, without the intervention of drugs.

The objects of the research were as follows: 1) to ascertain the tendency shown by *P. cynomolgi* to produce relapses in infections caused by serial passages of infected blood from monkey to monkey; 2) to establish whether the resulting relapses could be considered in relation to the coexistence of an hepatic endoepithelial cycle.

From the results obtained, it is evident that *P. cynomolgi*, inoculated with blood, produces in monkeys a primary attack varying in the present experiments from 10 to 59 days. In five out of 21 monkeys the length of the primary attack was from 36 to 59 days. This period, quite unusual in plasmodial infections, seems to indicate that the immunological process leading to the parasitolytic crisis is difficult to produce in this species of plasmodium. Also the fact that the same five monkeys showed relapses even after these very long primary attacks seems to indicate the low level of immunity produced in the host. Relapses after the end of the primary attack were frequent

during the entire period of observation (until 28 weeks after the end of the primary attack).

It is also evident that relapses may appear at any time during the period of observation, with more frequency at the 2nd–4th week, at the 15th–16th and at the 27th–28th, after the end of the primary attack. Out of 21 experimental monkeys, one died during the primary attack; two died respectively two and three weeks after the end of the primary attack; all the other monkeys, which survived for a longer time, had relapses.

In four monkeys (nos. 1, 2, 4, 5) Dr. Gramiccia examined sections of fragments of liver tissue removed by biopsy. A first biopsy of monkey no. 1 was made during the primary attack, at the 10th week after inoculation, and a second during a relapse, at the 13th week after inoculation. Monkey no. 2 had two biopsies during two relapses at the 8th and 22nd weeks respectively. A biopsy of monkey no. 4 was made during the primary attack, in the 2nd week after inoculation. Finally, monkey no. 5 had a biopsy during a relapse at the 17th week after inoculation.

All the biopsies made either during the primary attack, or during a relapse, gave negative results for hepatic endoepithelial forms.

DISCUSSION

In these experiments it has been shown that two different mammalian plasmodia, *P. malariae* and *P. cynomolgi* are capable of producing relapses, even after many passages from mammal to mammal by means of blood inoculation. It is thus demonstrated that inoculation of endoerythrocytic forms, instead of sporozoites, is sufficient to give infections which produce relapses.

In the present state of knowledge, there is no evidence that hepatic endoepithelial forms are produced in mammalian malaria, when the infection is obtained through blood inoculation. We do not know whether in mammalian plasmodia the endoerythrocytic parasites, introduced in a new host, are able to produce hepatic endoepithelial forms in the same manner in which endoerythrocytic parasites in *P. gallinaceum* are able to produce endohistiocytic forms. Hepatic endoepithelial forms have not been observed in the present experiments either during the primary attack or during relapses. These negative results are not sufficient to give scientific proof of their absence in blood-inoculated mammals: in any case their eventual presence is still to be demonstrated.

Since relapses are present in plasmodial infections of mammals even after a long series of passages all made by blood inoculation, it is difficult to support the opinion that relapses are exclusively dependent on the presence of hepatic endoepithelial forms. To prove such a statement it would be necessary first to demonstrate the continuous presence of endoepithelial forms in blood-inoculated mammals, and subsequently to demonstrate that relapses are dependent on these forms alone.

In the present state of knowledge, the interpretation of the origin of relapses given by Bignami still remains the most satisfactory. Not a single fact stands against this interpretation, which is the only one explaining all of the observed facts.

CONCLUSIONS

1. *Plasmodium malariae* maintains the property of producing long-term relapses even after at least 34 inter-human passages, all made through blood inoculation.

2. *Plasmodium cynomolgi* maintains the property of producing relapses after 12 passages from monkey to monkey, all made through blood inoculation. Three monkeys out of 21 died during the primary attack, or immediately after; the other 18 all showed relapses.

3. In blood-inoculated monkeys no hepatic endoepithelial forms have been found, either during the primary attack or during relapses.

4. The opinion that relapses should be exclusively dependent on the presence of hepatic endoepithelial forms is difficult to support. It would be necessary first to demonstrate the presence of hepatic endoepithelial forms in blood-inoculated mammals, and subsequently to demonstrate that relapses are dependent on these forms only. In the present state of knowledge, Bignami's interpretation of the mechanism of relapses still remains the most satisfactory.

5. Out of 19 monkeys infected with *P. cynomolgi*, 14 monkeys had a primary attack of 10–24 days; the other five showed an unusually long primary attack of 36–59 days. It is suggested that the immunological process leading to the parasitolytic crisis is difficult to produce in this species of plasmodium, and that only a low level of immunity can be normally reached by the host.

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OBSERVATIONS ON THE MECHANISM OF BLACKWATER FEVER

An Experimental Study in the Monkey

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The theories advanced to explain blackwater fever are like flowers—today they are present in all their beauty—tomorrow they begin to fade. Many of these theories are discussed in the publications by Boyd (1949), Maegraith (1948), and Russell and associates (1946). One factor that frequently appears in these is "hemolysis." Plehn (1920) regards an attack of hemoglobinuria in malaria as a condition analogous to anaphylactic shock, and he believes that a hemolytic process does not arise in the spleen but in the kidney when blood comes in contact with hypotonic urine which exerts an hemolytic effect on the red cells. Gear (1946) has suggested that in blackwater fever the spleen may act as a reservoir for autohemolysins. These may be forced into the general circulation during splenic contraction to produce the sudden onset of hemolysis that promotes an attack of hemoglobinuria. Belding (1942) thinks that the red blood cells are reduced in number by both the destructive action of the parasites and the hemolytic activity of the reticulo-endothelial cell. Maegraith, Findley and Martin (1943) believe that the serum normally contains an inhibitory substance that prevents the tissues (spleen, liver and lung) from causing lysis of the red cells. The cause of blackwater fever then is the lack of this inhibitory substance. Dudgeon (1920) thought that he demonstrated a hemolytic substance in the spleen and in the urine during blackwater fever that was not present either in normal urine or in cases of malaria when blackwater fever was not present. It has been postulated that over production of both lactic and sarcolactic acid is the cause of blackwater fever in malaria (Blacklock and Macdonald 1928). Lysolecithin, a powerful *in vitro* hemolytic agent, has been discussed as a possible agent in blackwater fever by Foy and Kondi (1943). The occurrence of defective erythrocytes in malaria that are readily lysed has been thought to contribute to hemoglobinuria (Maegraith 1948) (Gear 1946) (Vint 1941). It would appear, however, at this time that there is unsufficient evidence to establish the fact that a "hemolysin" is a significant factor in the production of the hemoglobinuria that occurs in malaria.

The sudden onset of hemoglobinuria in malarial infections has been frequently observed and appears to be an important factor in the pathogenesis of blackwater fever. Gear (1946) postulates that splenic contractions liberate autohemolysins that account for the sudden onset of hemolysis and this promotes an attack of blackwater fever. Another theory is that the contraction of the spleen accentuates the

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already existing anoxemia and more lactic acid is formed resulting in greater hemolysis (Blacklock 1928). Charters and associates (1945), in discussing their cases of blackwater fever, say "These observations show that blackwater fever is very intimately associated with a contraction of the spleen . . . contraction of the spleen which is often brought on by stimulation of the sympathetic nervous system, is an essential factor in the causation of blackwater fever." These investigators believe that the splenic contraction is the cause of the hemolytic process and suggest that the greater splanchnic nerves should be infiltrated with absolute alcohol to paralyze splenic contraction and thus eliminate the cause of blackwater fever.

The significance of splenic contraction in the mechanism of blackwater fever also is indicated by the precipitation of hemoglobinuria by certain drugs. As many as four attacks have occurred in one patient following the administration of quinine (Fairley and Murgatroyd 1940). There was an interval of time between each of these. Following effective treatment with another antimalaria drug and the disappearance of splenomegaly, quinine no longer precipitated hemoglobinuria in this patient. Kitchen and Sadler (1945), Deeks and James (1949), and Charters et al (1935) made a similar clinical observation with quinine. The latter investigators think that as long as the spleen remains large and quinine is given a person is subject to an attack of blackwater fever. Gear (1946) has listed the precipitating causes of blackwater fever in order of their frequency as quinine, chill, exertion and violent emotion. Each of these cause a contraction of the spleen (Gear 1946). In support of the role of splenic contraction in precipitating an attack of blackwater fever is the observation made in East Africa that a large dose of phenobaritone is effective in arresting hemoglobinuria (Maegraith 1944). Smooth muscle activity is depressed in varying degree by large doses of barbiturates and the contractor response of epinephrine is diminished (Sollmann 1948).

The role of the spleen in the mechanism of hemoglobinuria was demonstrated by Krishnan and associates (1933) in their study of *Plasmodium knowlesi* infection in the monkey. They observed that when normal *Macacus rhesus* monkeys were splenectomized and then inoculated with *P. knowlesi*, hemoglobinuria occurred in 100 per cent of the animals. Hemoglobinuria also occurred in 100 per cent of a group of splenectomized *M. rhesus* monkeys with a history of a previous *P. knowlesi* infection. Hemoglobinuria occurred in only 28.6 per cent of a non-splenectomized group of monkeys. Hemoglobinuria occurred in only 15 per cent of a non-splenectomized group treated with quinine. It is easy to see from these experiments that the incidence of hemoglobinuria is extremely high in the splenectomized monkeys when compared with a non-splenectomized group.

To study the mechanism of blackwater fever in malaria, we have produced splenic enlargement in the monkey and then caused its contraction by adrenalin with a resulting hemoglobinuria.

MATERIALS AND METHODS

The *M. mulatta* monkeys used in this study were inoculated with *P. knowlesi*. The parasitemia was followed by counting the number of parasitized erythrocytes per 500 red cells in blood smears obtained from the marginal veins in the ears. These

were stained with a combination of Wright's and Giemsa's stains. Standard techniques were used for the erythrocyte counts.

Hemoglobinuria was determined by macroscopic examination of the urine. The monkeys were carefully observed to determine the time of voiding following the injection of adrenalin hydrochloride. This drug was given intramuscularly in amounts varying from 0.5 to 1.0 cubic centimeters. Sterile techniques were used in transplanting the spleen into the subcutaneous tissue of the left side of the abdomen. The blood supply was not disturbed by this procedure. The size of the spleen was determined by palpation and measuring the organ in three planes at frequent intervals during the experiments. Quinine dihydrochloride was given intravenously, intraperitoneally and intramuscularly to some of the monkeys as shown in the different experiments.

EXPERIMENTAL

Hemoglobinuria in non-fatal P. knowlesi *infections*

The typical course of *P. knowlesi* infection in *M. mulatta* monkeys is well known (Knowles and Das Gupta 1932). The progressive increase in the parasitemia is accompanied by a corresponding decrease in the number of erythrocytes. Death ultimately results from anoxemia that is secondary to the anemia produced by the plasmodia (Rigdon and Stratman-Thomas 1942) (Rigdon 1948). Hemoglobinuria is a frequent complication that occurs during the terminal phase of this infection (Knowles and Das Gupta 1932) (Napier and Campbell 1932). Splenomegaly is always present at the time of death in *P. knowlesi* infected monkeys. However, according to Coggeshall (1937), the spleen may not increase in size during the twenty-four hours preceding death. This final cessation of the increase in size of the spleen probably is correlated with the great cellular destruction which accompanies the terminal stages of the infection in *M. mulatta* monkeys.

During our studies of *P. knowlesi* infection in *M. mulatta* monkeys, hemoglobinuria usually has occurred as a terminal event in fatal cases. The fact that an occasional monkey has shown hemoglobinuria and survived following therapy has contributed much to our concept of the pathogenesis of blackwater fever. Figure 1 shows the time of occurrence of hemoglobinuria in Monkey 28. This hemoglobinuria occurred following a rapid increase in the degree of parasitemia. It also ceased rapidly following the administration of quinine. It is important to note in this monkey that the hemoglobinuria was not present after the third and fourth injection of quinine. We have not observed the occurrence of hemoglobinuria either following the administration of quinine in *P. knowlesi* infected *M. mulatta* monkeys or in normal monkeys given one or more doses of quinine after varying intervals. It is easy to understand from a review of the literature how the idea originated that hemoglobinuria probably was the result of quinine therapy.

Hemoglobinuria produced by adrenalin in P. knowlesi *infected monkeys*

Four monkeys, 45, 50, 51 and 52, infected with *P. knowlesi* were given adrenalin and each developed hemoglobinuria. Figure 2 is the record on Monkey 45. Hemo-

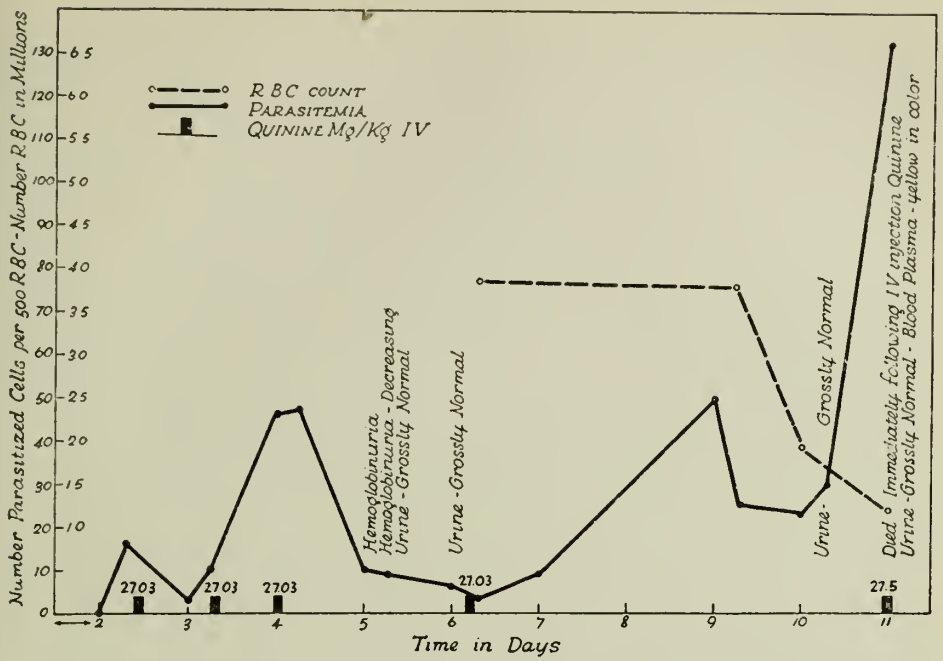

FIG. 1. Monkey 28. The hemoglobinuria occurred on the fifth day following the increase in parasitemia. This has been an infrequent complication occurring at this time during *P. knowlesi* infection in the *M. mulatta* monkey.

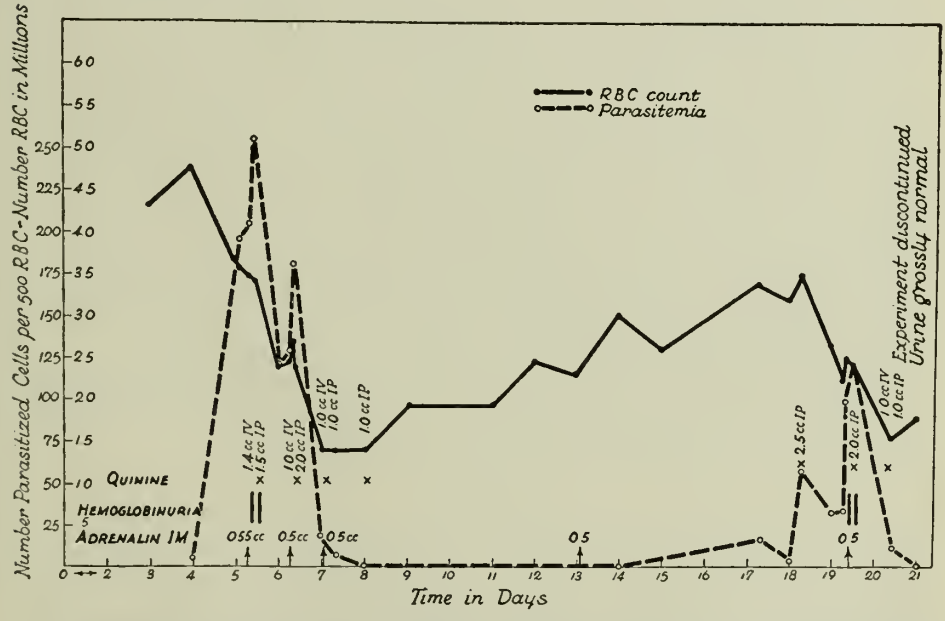

FIG. 2. Monkey 45. Hemoglobinuria occurred following the intramuscular injection of adrenalin on the fifth and nineteenth day of the experiment. No hemoglobinuria occurred following the injection of adrenalin on the sixth, seventh and thirteenth. Note the rise in parasitemia preceding the occurrence of hemoglobinuria on both occasions.

globinuria occurred after two of the five injections of adrenalin. The parasitemia apparently increased immediately following the adrenalin in three of the five experiments. Monkey 52 was given one injection of adrenalin and hemoglobinuria occurred thirty-five minutes later for only a short time.

The spleen was transplanted beneath the skin of the abdomen in Monkeys 50 and 51 in an attempt to follow the contraction of this organ following the intramuscular injection of adrenalin. The amount of the contraction, however, was so slight that it was difficult to determine it by palpation. Hemoglobinuria did occur, however, in Monkey 52 once following one of the two injections of adrenalin. This experiment was repeated in Monkey 51. He had only a moderately severe parasitemia on the sixth experimental day. The spleen was definitely larger (4.2 x 3.2 x 1.5 cm.) than normal (3.3 x 2.9 x 1.1 cm.). One cubic centimeter of adrenalin hydrochloride was given intramuscularly and five minutes later the spleen measured 4 x 3 x 1.1 centimeters. Three hours later the monkey was voiding slightly blood tinged urine and five hours after the adrenalin the urine appeared as pure hemoglobin. The parasitemia rapidly increased and the red cells decreased in number. Hemoglobinuria continued until death occurred twenty hours following the time of the injection of the adrenalin. It may have been only a coincidence that hemoglobinuria occurred so soon after the injection of adrenalin in this animal. However, such should be considered as a possible precipitating agent. The spleen did contract following the injection of the adrenalin in this monkey.

OBSERVATIONS ON HEMOGLOBINURIA IN RE-INFECTED MONKEYS

It has been suggested that blackwater fever is a manifestation of sensitivity (Fernau-Nunez 1936), (Butts 1945) (Oliver-Gonzalez 1944) (Cleland 1909). To study this possibility in the monkey, two animals were infected with *P. knowlesi.* They were treated with human blood (Rigdon and Breslin 1949) and quinine and recovered. Twenty months later they were reinoculated with *P. knowlesi* and both developed a low parasitemia. Hemoglobinuria, however, did not occur in either of these two animals.

In our study of the effect of phenylhydrazine hydrochloride on *P. knowlesi* infection in the *M. mulatta* monkey (Rigdon *et al* (In press)), several of the animals had a spontaneous recurrence of their infection within a period of one to four months. However, hemoglobinuria was never observed in any of these monkeys.

From these observations in the monkey, it would appear that hemoglobinuria is not likely to be a manifestation of sensitivity. Furthermore, as is well known, many monkeys show hemoglobinuria with their original infection.

DISCUSSION

Our experimental observations suggest that the destruction of the red cells by the plasmodia is the basis for the cellular debris and liberation of pigments that occur in malaria. These pigments and debris are readily removed from the circulating blood by the reticulo-endothelial system. Much of it accumulates within the spleen and ultimately there may be splenomegaly. With a progressive increase in the num-

ber of parasites, enough red cells may be destroyed within a specific interval that the debris and pigments can no longer be removed from the plasma by phagocytosis. With a high plasma concentration of these pigments, filtration through the rena glomeruli occurs, resulting in hemoglobinuria. Yuile (1942) has said, "In dogs and presumably other mammals the rate of hemoglobin excretion above a threshold level is directly proportional to its concentration in the plasma."

The primary process, therefore, in malaria that may result in blackwater fever is a disproportion between the rate of destruction of red cells and the rate of removal of the residue by the reticulo-endothelial system. In this connection, it must be remembered that the spleen enlarges to accommodate additional pigments and debris. Much of this debris may not be incorporated at first within the cytoplasm of the phagocytic cells. At this time during the disease any process causing the spleen to contract may squeeze the cellular debris and pigments out into the circulating plasma. With the plasma concentration then greatly increased, filtration through the renal glomeruli is the only major route by which it can be eliminated. When this does occur, the urine shows the presence of this excess pigmentation and the process is referred to as hemoglobinuria. Histologic studies of the kidneys from monkeys with blackwater fever have shown that some of the pigments that filter through the glomeruli are present in the epithelial cells of the convoluted portion of the renal tubules (Rigdon 1949).

Factors that affect the process of filtration in malaria apparently are the same as those governing all processes of renal filtration. Some of the pigments resulting from this infection are filtrable, while some of the debris may not pass through the glomeruli. Hemoglobinuria may also either decrease or subside as a result of a diminution in the level of the blood pressure. This may readily occur in those animals that develop a severe anemia with an accompanying anoxia. The effect of anoxia on the myocardium and kidneys results in inefficient renal filtration.

There does not appear to be any need to speculate upon the presence of a "hemolysin" to explain the mechanism of hemoglobinuria in monkey malaria since such apparently has never been proven. When enough red cells have been destroyed by the parasites and the spleen has become enlarged as a result of the accumulation of these pigments and debris, the stage is set for the occurrence of hemoglobinuria. A contraction of the spleen from any cause may now result in hemoglobinuria. The continued destruction of erythrocytes by malarial parasites when the spleen is already large may result directly in hemoglobinuria. Adrenalin has been shown to produce hemoglobinuria in the monkey infected with *P. knowlesi*. At no time in the monkey have we observed hemoglobinuria following the injection of quinine. However, we would not be surprised if such did occur since quinine given intravenously may produce convulsions (Rigdon and Ruskin 1949). Hemoglobinuria might also occur in malarial infected monkeys following contraction of the spleen produced by excessive exercise and fear associated with handling. As previously stated, hemoglobinuria has been observed in man following quinine therapy (Fairley and Murgatroyd 1940), and attacks have been observed following violent emotions (Gear 1946). The inhibition of the attacks of blackwater fever with barbiturates lends support to the significance of splenic contraction (Maegraith 1944).

There is no evidence in our study of hemoglobinuria in the monkey to support the idea that this complication is related to sensitivity. Monkeys with a recurrence of malaria have not been observed to develop this complication. It is reasonable to think that this might occur, however, if the second attack is characterized by a rapid and high degree of parasitemia producing an excessive amount of pigment and cellular debris similar to that occurring during an original infection.

Others have discussed the role of splenic contractions in the production of hemoglobinuria. However, they have also considered a hemolysin as being present (Gear 1946). It is our opinion that the splenic contraction only "squeezes" unattached pigments out into the circulating blood and produces an excessive concentration in the plasma which is eliminated by filtration through the renal glomeruli. Anything that produces contractions of a large spleen in malaria may, therefore, result in hemoglobinuria.

SUMMARY

Hemoglobinuria frequently occurs as a terminal event in *M. mulatta* monkeys infected with *P. knowlesi*. In a few monkeys, hemoglobinuria has been observed to follow a rapid increase in the degree of parasitemia. Some of these latter animals have been treated with quinine and have survived the malarial infection. The hemoglobinuria subsided following therapy. Monkeys with malaria have been given adrenalin hydrochloride and hemoglobinuria occurred. Following treatment with quinine these monkeys recovered from both the hemoglobinuria and the acute malarial infection.

It is suggested that hemoglobinuria in malaria is the result of the excessive destruction of erythrocytes by the plasmodia. The resulting pigments and debris are normally phagocytized by the cells of the reticulo-endothelial system. The spleen becomes enlarged because of its phagocytic activity. If destruction of the erythrocytes by the plasmodia continues at a rapid rate, the reticulo-endothelial system is unable to remove these pigments. Thus, a high plasma level results. When the concentration of pigments in the plasma is greater than the renal threshold value, the excess is filtered through the glomeruli into the urine, producing the condition recognized as hemoglobinuria.

An attack of hemoglobinuria may be produced by contracting the spleen with adrenalin. The action of this drug is to "squeeze out" pigments and debris from the spleen into the circulation. The blood plasma concentration then will be increased over the normal renal threshold value with a resulting filtration of these pigments into the urine. To precipitate an attack of hemoglobinuria by adrenalin, it is necessary that many red cells should have been recently destroyed by the plasmodia and the spleen must be enlarged. The phenomenon of hemoglobinuria results from a disproportion between the rate of destruction of the erythrocytes and the ability of the reticulo-endothelial system to remove the pigments from the plasma. There is nothing in this study to suggest the presence of a "hemolysin" to account for the hemoglobinuria.

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THE PRECIPITIN TECHNIQUE FOR DETERMINING SPECIES OF HOST BLOOD IN MOSQUITOES— MODIFICATIONS AND IMPROVEMENTS

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In the course of testing the blood meals of mosquitoes by the method of Arnold, Simmons, and Fawcett (1), it became apparent that the results were not always dependable. Accordingly, all the phases of the test were studied using mosquitoes which were known to have fed on certain anmals in order to measure the accuracy of the test. As a result of this study, modifications in the equipment and procedures were instituted in this laboratory. It is the purpose of this paper to describe the precipitin test as it is now carried out in the Serology Laboratory of the Communicable Disease Center.

METHODS

Mosquitoes used for the study were prepared at the insectary of the Emory Field Station, Newton, Ga. Mosquitoes of known feedings were crushed on individual pieces of filter paper and sent to the laboratory.

Preparation of testing reagents

1. Antigens: Control antigen was prepared by diluting normal serum of the desired species of animal 1 in 500 with physiological saline. Test antigen, i.e. blood meal, was extracted from mosquitoes fed on known species of animals. Each engorged mosquito was placed in 1.5 ml of saline and stirred slightly with an applicator to remove the blood from the mosquito and filter paper. It was found that a 1 in 500 dilution of normal serum was approximately equivalent to the dilution of blood obtained if the engorged mosquito was placed in 1.5 ml saline. The quantity of blood present in an engorged mosquito was studied by Frobisher (3).

2. Antisera: The antisera or precipitin antibodies were prepared by injecting normal serum of the desired species of animal into rabbits intravenously. The injections were given at two-day intervals, starting with 0.5 ml on the first day, followed by 1.0 ml, and finishing with 1.5 ml. On the first day of the fourth week after the last injection, the rabbits were desensitized by injecting 0.1 ml of serum intradermally. After 30 minutes, an intravenous "booster" injection was made of 0.5 ml of serum. A small amount of blood was drawn after 48 hours. If the titer of the sample of blood was 1 in 8 or higher, the rabbit was exsanguinated by puncture of the femoral vein shortly after the preliminary titration.

In the event that the titer was not satisfactory, the rabbit was given a second injection one week after the first booster injection and retested. The rabbits were

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not given more than three booster injections. An animals that had failed to respond was not used for further injections. Antisera from rabbits used repeatedly for the preparation of antisera were found to react nonspecifically. After booster injections, serum was checked carefully for specificity. Satisfactory antisera were diluted 1 in 2 with glycerine and stored in the regrigerator.

Antisera were titrated against a standard 1 in 500 dilution of antigen (normal serum). The antiserum was diluted to 1:4, 1:5, 1:8, 1:16, and 1:32, using glycerine-saline solution[2], as the diluent. Of each dilution of antiserum, 0.1 ml was transferred to 4 x 50 mm tubes with a capillary pipette and layered carefully with 0.1 ml of 1:500 dilution of the homologous antigen. The tubes were incubated at room temperature for 30 minutes. The presence of a white, fleecy precipitin ring at the zone between the antigen and antiserum was indication of a positive reaction. Serum which gave a titer of 1:4 has been used, but a titer of 1:8 or higher was preferred. Satisfactory antisera must have a sufficient titer and must react only with the antigen used in their preparation.

TABLE 1

*Determination of titer and test for specificity**

DILUTION OF ANTISERUM	ANTIGEN: 1:500 DILUTION OF NORMAL SERUM				
	Human	Equine	Bovine	Porcine	Avian
1:4	−	+	−	−	−
1:6	−	+	−	−	−
1:8	−	+	−	−	−
1:16	−	+	−	−	−
1:32	−	−	−	−	−

* Equine antiserum was used in this titration.

The specificity of each dilution of antiserum used was tested against the serum of various animals in addition to the homologous animal. In this laboratory, the determination of specificity was made with human, equine, bovine, procine, and avian sera. The antiserum was diluted as mentioned above. Of each dilution of the antiserum 0.1 ml was placed in 4 x 50 mm. test tubes and layered carefully with 0.1 ml of the 1:500 dilution of each antigen, using capillary pipettes. The tests were incubated at room temperature for 30 minutes and observed for precipitin reactions. As a rule, several antisera were titered and tested for specificity at the same time. These tests were set up as shown in Table 1. A titer of 1:16 with no cross reaction was accepted as indicating a good antiserum.

Equipment

In addition to the problem of preparing sufficiently potent and specific antisera, it was found that the equipment was the next most important factor in routine op-

[2] Formula:

Glycerine	332 ml
Distilled H_2O	660 ml
NaCl	8.5 gm

eration of the precipitin test. Improvements made in the equipment and procedures previously described by Arnold, Simmons, and Fawcett (1) are described as follows:

1. *Mixing Chamber.* It was difficult to be certain that mixing chambers made of metal and painted with enamel were chemically clean. Accordingly, a plastic mixing vessel was designed with 10 separate, molded, shallow, triangular chambers. (Fig. 1.)

2. *Tube Holder.* The capillary tube hold or "card" as described by Arnold, Simmons, and Fawcett (1), was undesirable because of its weight and the large breakage factor. A new capillary tube holder was originated consisting of one piece of plastic 3 x $\frac{3}{4}$ inches in size and $\frac{1}{8}$ inch thick. Five parallel slots equal to the outside diameter of the capillary tubes were cut $\frac{1}{16}$ inch deep at $\frac{1}{2}$ inch intervals into the plastic strip.

FIG. 1. Plastic mixing vessel and paper punch

Capillary tubes were fastened into the slots with sealing wax. (Fig. 2.) Broken capillary tubes were easily replaced by melting the sealing wax and replacing the broken tubes.

3. *The Reading Light.* The viewing box as described in Boyd's "Malariology" (2) was cumbersome and unwieldy. It was found that an ordinary hand magnifying lens suspended in front of a fluorescent table lamp was satisfactory. The precipitin test was read against a dark background obtained by placing a piece of black cardboard behind the light. A positive precipitin reaction is shown in the second capillary tube from the left. (Fig. 3.)

4. *Washing.* The capillary tube holders or cards were washed thoroughly by means of a device for forcing water through each capillary tube simultaneously. (Fig. 4.)

5. *Drying.* It has been demonstrated that the presence of traces of water which were not removed from the capillary tubes after washing gave false reactions. For

FIG. 2. Capillary tube holder

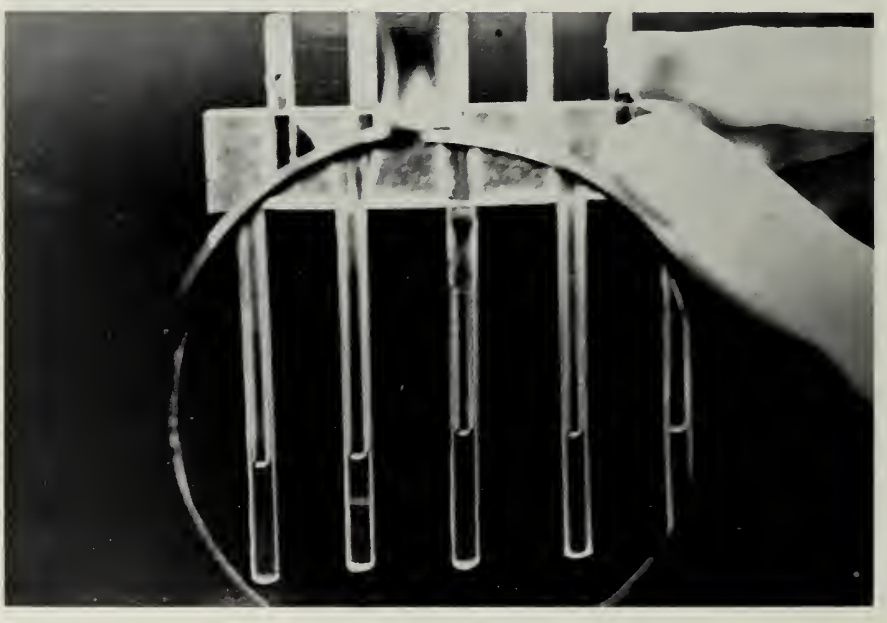

FIG. 3. Precipitin test reading arrangement

this reason, each tube was dried individually by means of an aspirator suction pump. (Fig. 5.)

FIG. 4. Capillary tube washing apparatus

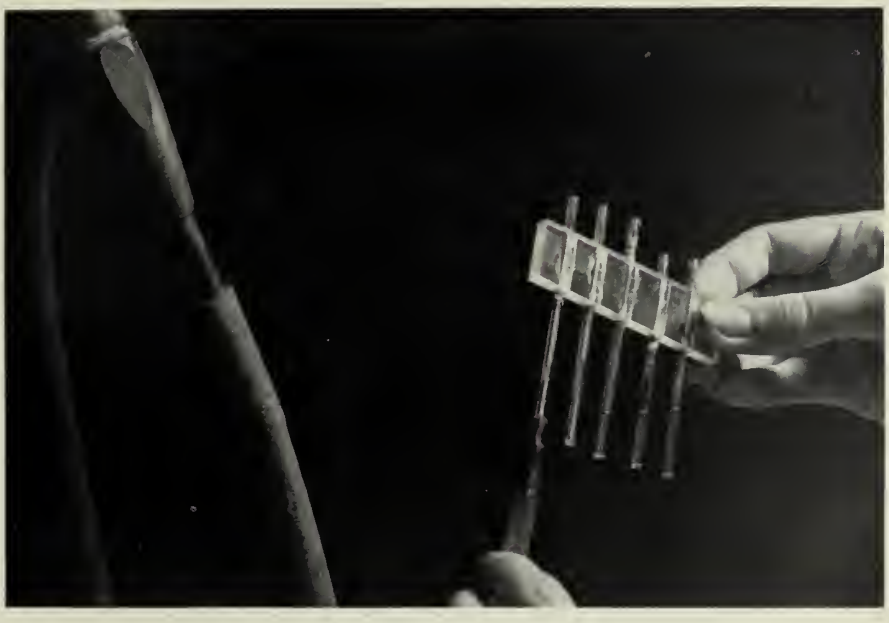

FIG. 5. Aspirator pump drying technique

Testing of blood meals of mosquitoes

Both antigen controls and known mosquito controls were prepared by filling vials (7 x 35 mm) with 1:500 dilutions of each of the five different normal sera used as

antigens. The 1:500 dilutions of these antigens (using saline as the diluent) were prepared each week. The antisera were diluted with the glycerine-saline solution according to their determined titers and placed in vials (7 x 35 mm).

Tests were carried out with five antigens at a time using the capillary tube holder previously described. The antigens were drawn up into the capillary tubes by capillary action to the height of about $\frac{1}{2}$ inch. Approximately half of the volume was removed by blotting the tubes on a blotter made of cotton moistened with physiological saline and overlaid with a large sheet of filter paper. The capillary tubes then were dipped into the five vials of antisera (each tube entering a different vial) and the volumes of the liquids in the tubes were restored to the original height.

TABLE 2

Comparison of results obtained with Aedes aegypti *and* Anopheles quadrimaculatus *mosquitoes fed on human, equine, bovine, and avian hosts*

HOST AND SPECIES OF MOSQUITO	NUMBER TESTED	CORRECT	INCORRECT	NEGATIVE	UNSATISFACTORY*
Human					
A. quad.	11	11	0	0	0
A. aeg.	34	32	0	2	0
Equine					
A. quad.	22	18	0	4	0
A. aeg.	23	14	0	9	0
Bovine					
A. quad.	33	31	0	2	0
A. aeg.	10	3	0	7	0
Avian					
A. quad.	26	22	0	4	0
A. aeg.	61	1	2	37	21

* Unsatisfactory results are recorded upon the observation of a white haziness throughout the capillary tubes.

The tubes were incubated at room temperature for 30 minutes and read. This was repeated with mosquitoes of known feedings.

Each mosquito with its dried blood meal, was cut out of the filter paper triangle on which it was sent to the laboratory, using a large-holed hand punch (Fig. 1). Each paper with the blood meal was placed in a separate chamber of the plastic mixing vessel. One and one-half ml of saline were added to each chamber and held at room temperature for 30 minutes. The five tubes of a capillary tube holder first were dipped in a mosquito saline mixture, after which each of the five tubes was dipped into a different antiserum as described above.

RESULTS WITH MOSQUITOES FROM KNOWN FEEDINGS

Using the equipment and procedure described above, tests were made with mosquitoes fed on known animals. The results are shown in Table 2.

In Table 2 it is seen that better results are obtained with *A. quadrimaculatus* than

with the *A. aegypti* mosquitoes. This is especially true when the results are compared with those obtained with the *A. aegypti* fed on the avian hosts. However, an examination of the avian fed *A. aegypti* showed that the blood meal of all of those giving negative results was very scanty, or was hemolyzed and digested. On the other hand, all the *A. quadrimaculatus* generally consumed a large blood meal.

Results of tests with mosquitoes from known feedings selected according to the condition and quantity of the blood meal are shown in Table 3.

It may be observed in table 3, that relatively undigested blood meals and fully engorged mosquitoes gave the best results. Under the conditions of the insectary where these specimens were obtained, digestion and hemolysis took place within 17 hours among some of the mosquitoes. These data were presented to explain the cause of negative results and the necessity for selecting adequate specimens. Although

TABLE 3

Comparison of mosquitoes selected according to the condition of the blood meal

HOST AND SPECIES OF MOSQUITO	CONDITION OF BLOOD MEAL	NUMBER TESTED	CORRECT	INCORRECT	NEG.	UNSATISFACTORY†
Avian (*A. aegypti*)	Fully engorged. (17 hrs.)*	29	29	0	1	0
Avian (*A. aegypti*)	Poorly fed (17 hrs.)*	29	24	0	5	0
Avian (*A. aegypti*)	Digested and hemolyzed (17 hrs.)*	18	12	0	6	0
Avian (*A. quad.*)	Poorly fed (2 hrs.)*	6	2	0	4	0
Avian (*A. quad.*)	Fully engorged; no digestion (2 hrs.)*	6	6	0	0	0

* Indicates the hours lapsing between the ingestion of the blood meal and the preparation of the filter paper specimen.

† Unsatisfactory results are recorded upon the observation of a white haziness throughout the capillary tubes.

Frobisher (3) showed that good reactions sometimes were obtained with *A. aegypti* tested two days after ingestion, negative results also were obtained with some mosquitoes tested earlier.

SUMMARY

Although the precipitin test itself is a relatively simple procedure, many problems have developed when carried out on 20,000 mosquitoes annually. The illustrations and descriptive data are presented here to help others who find it necessary to do similar testing. The importance of preparing high-titer, specific antisera cannot be over emphasized. In addition to the sera, the capillary test tubes, in which the precipitin test is carried out, are of great importance. These must be chemically clean and dry at the time of setting up the tests.

As in other serological tests, good results are largely dependent upon the specimen. Unsatisfactory results are obtained if complete digestion of the blood meal has occurred. Best results were obtained with fully engorged mosquitoes collected within 17 hours after feeding.

SUMARIO

Aunque la prueba de precipitinas es un procedimiento relativamente simple en sí, se han presentado muchos problemas cuando se han llevado a cabo con 20,000 mosquitos anualmente. Se presentan aquí ilustraciones y datos para facilidad de los que necesiten hacer pruebas similares. Debe hacerse hincapié en la importancia de preparar antisueros específicos de alto título. Además del suero, los tubos capilares en los cuales se hacen las pruebas son de gran importancia. Estos deben estar químicamente limpios y secos al comenzar las pruebas.

Como en todas las pruebas serológicas los buenos resultados dependen grandemente del especímen. Se obtendrán resultados desfavorables si ocurre digestión completa del medio sanguíneo. Los mejores resultados se obtuvieron con mosquitos completamente hartos de sangre dentro de las 17 horas posteriores a la comida.

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THE COMPARATIVE SUSCEPTIBILITY OF *ANOPHELES QUADRIMACULATUS* AND TWO STRAINS OF *ANOPHELES ALBIMANUS* TO A PANAMA STRAIN OF *PLASMODIUM FALCIPARUM*[1]

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This report is one of a series from these laboratories comparing the suceptibility of American *Anopheles* to various strains of *Plasmodium*. The present study is concerned with the susceptibility of *Anopheles quadrimaculatus*, a Panama strain of *A. albimanus* and a strain of *A. albimanus* from the Florida Keys to a Panama strain of *Plasmodium falciparum*.

An earlier report in this series (Eyles and Young, 1950) has recorded observations on the susceptibility of *A. quadrimaculatus* and *A. albimanus* from Panama to a United States strain of *P. falciparum*. Several other investigators have noted differences in susceptibility of *A. albimanus* and *A. quadrimaculatus* when exposed to infection with several strains of *Plasmodium* (Boyd *et al.*, 1938, 1940; Young *et al.*, 1946).

METHODS

Two of the strains of *Anopheles* used in this study were the same as those used by Eyles and Young (1950). These were *A. quadrimaculatus*, designated in this laboratory as the Q-1 strain, and *A. albimanus* domesticated by Rozeboom (1936) from Panama and designated by us as the A-2 strain. The other strain of *A. albimanus* used was domesticated from the Florida Keys, by Burgess (1950) and is designated as the A-3 strain.

The *P. falciparum* used is designated in this laboratory as P. F.-6, and was recovered from a mestiza of El Limon, Transisthmian Highway, Panama, in 1948.

The procedures consisted of feeding about 100 of each species of mosquito simultaneously on a patient showing adequate gametocyte densities. The engorged specimens were maintained in an insectary at 74–78° F. and were dissected and the oöcysts counted 11–13 days, usually 12, after the blood meal. This period of incubation gave assurance that the oöcysts were sufficiently large to be counted accurately but sporozoites were not yet present in the salivary glands. An average of 34 individuals per lot was dissected.

It was not at all times possible to feed all three strains simultaneously; therefore, there are a number of feedings in which only two were fed at the same time (*A. quadrimaculatus* with Panama *A. albimanus* or the two strains of *A. albimanus*). In the results the three strains are considered in pairs. Mosquitoes of approximately the same age were used in each case.

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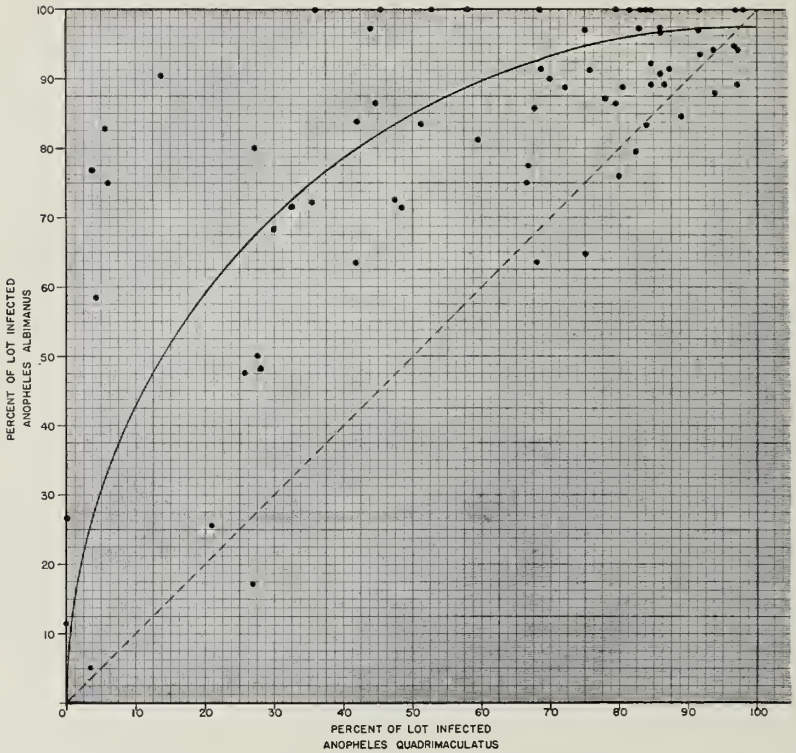

FIG. 1. A comparison of the percentage of mosquitoes infected when 70 paired lots of *Anopheles quadrimaculatus* and Panama *A. albimanus* were fed simultaneously on a Panama strain of *Plasmodium falciparum*. Each point represents a pair of lots and the curve was fitted by inspection.

TABLE 1

Summary of 70 paired feedings comparing the susceptibility of Anopheles quadrimaculatus *and* A. albimanus (*Panama strain*) *to a Panama strain of* Plasmodium falciparum

MOSQUITO STRAIN	NO. DISSECTED	PER CENT INFECTED	P VALUE†	AVERAGE RATIO OF MEAN OÖCYST COUNTS PER INFECTED GUT, *A. albimanus*/ *A. quadrimaculatus*
A. quadrimaculatus (Q-1)	2,497	62.1 ± 1.0	0.0001*	7.2 ± 1.2 (S.D.‡ = 9.2)
A. albimanus (Panama, A-2)	2,264	79.9 ± 0.8		

* Susceptibility greater for *A. albimanus*.

† Probability of the difference in proportion infected being due to chance.

‡ Standard deviation.

OBSERVATIONS

Comparative susceptibility of Panama Anopheles albimanus *and* Anopheles quadrimaculatus. Seventy pairs of feedings were done to compare the susceptibility of these

two vectors to a Panama strain of *P. falciparum*. In 59 instances there was a larger proportion of the coindigenous *A. albimanus* infected; in 11 instances the proportion was the same or lower in *A. albimanus*. Summarizing the data from the 70 lots (table

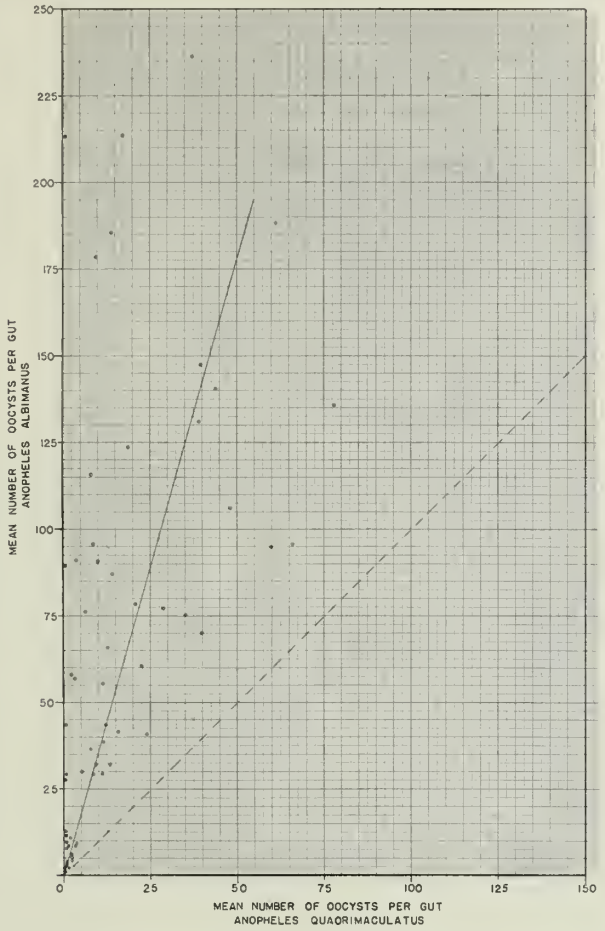

FIG. 2. A comparison of the intensity of infection as measured by mean oöcyst counts in 70 paired lots of *A. quadrimaculatus* and Panama *A. albimanus*.

Each point represents a pair of feedings and the curve was fitted by inspection.*

1), it is seen that 79.9 per cent of the *A. albimanus* mosquitoes became infected as compared with 62.1 per cent of the *A. quadrimaculatus* mosquitoes. This difference was found to be highly significant (P = 0.0001).

Figure 1 charts the proportions of the two species infected in 70 comparative feedings on the Panama strain of *P. falciparum*. It is obvious that when infection

* Six points are lost due to crowding at the lower end of the curve; also extreme high lots (six in number) are not plotted in order to reduce the size of the figure.

TABLE 2

A comparison of the proportions of Anopheles quadrimaculatus *and* Anopheles albimanus *mosquitoes susceptible to infection with a Panama strain of* Plasmodium falciparum *at varying levels of infection intensity as measured by oöcyst counts*

NO. OF OÖCYSTS PER GUT	*A. albimanus* (PANAMA)		*A. albimanus* (FLORIDA KEYS)		*A. quadrimaculatus* (UNITED STATES)	
	No. of Lots	Percentage of Individuals Infected	No. of Lots	Percentage of Individuals Infected	No. of Lots	Percentage of Individuals Infected
1– 10	17	60.6	13	63.5	35	46.1
11– 20	4	63.1	4	90.6	13	82.2
21– 40	9	86.3	5	92.1	8	83.0
41– 80	15	89.6	4	95.0	9	77.8
81–160	15	85.7	3	98.2	3	73.9
161–320	7	93.9	1	94.3	*	*
320 and over	4	100.0	2	96.3	*	*

* None of the *A. quadrimaculatus* lots averaged over 160 oöcysts per gut.

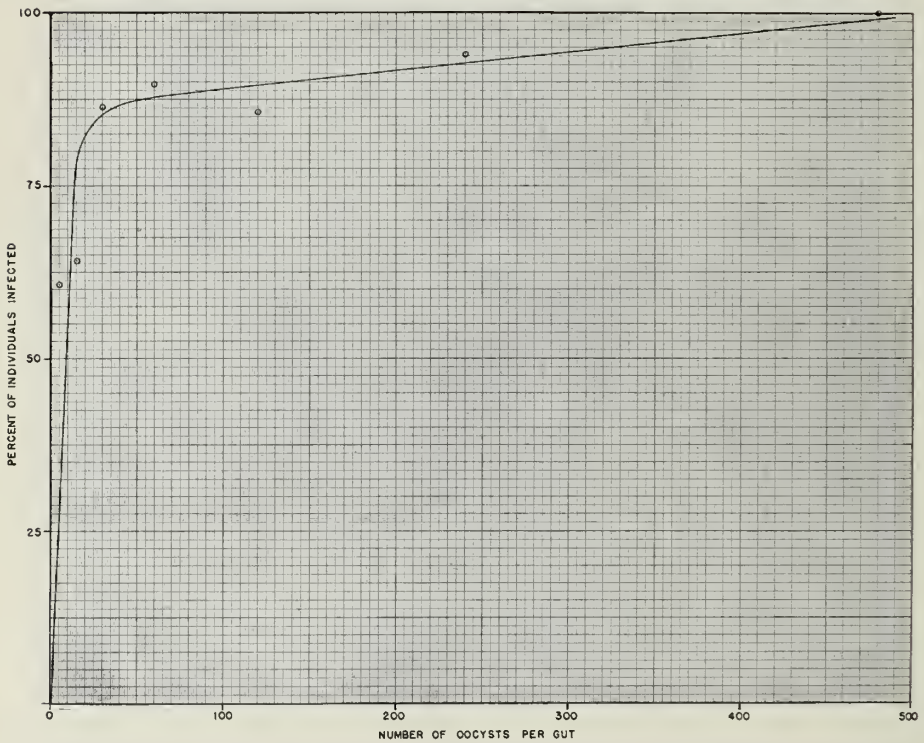

FIG. 3. Graph showing relationship between intensity of infection and percentage of individual mosquitoes infected for Panama *Anopheles albimanus* infected with a coindigenous strain of *Plasmodium falciparum*.

intensity becomes high there is less difference in the proportions infected of the two species; whereas, at low intensities of infection the *A. albimanus* lots had a much larger number of individuals infected.

In addition to comparisons of proportions infected of the two species, mean oöcyst counts were calculated for all lots. The ratio of these means for each pair of lots fed simultaneously was also calculated and the mean ratio derived (table 1). The Panama *A. albimanus* had an average of 7.2 times as many oöcysts per infected individual as did *A. quadrimaculatus*. This relationship is shown graphically in figure 2.

The data from the 70 feedings were also examined to determine if any fractions of the mosquito populations were refractory to infection with the particular strain of malaria being used. Table 2 indicates that as infection intensity increases the proportion of infected individuals also increases for both species. In the Panama *A. albimanus* the proportion seems to approach 100 per cent. This relationship is shown

TABLE 3

Summary of 27 paired feedings comparing the susceptibility of Anopheles quadrimaculatus *and* A. albimanus (*Florida Keys strain*) *to a Panama strain of* Plasmodium falciparum

MOSQUITO STRAIN	NO. DISSECTED	PER CENT INFECTED	P VALUE	AVERAGE RATIO OF MEAN OÖCYST COUNTS PER INFECTED GUT, *A. albimanus*/*A. quadrimaculatus*
A. quadrimaculatus (Q-1)	900	76.8 ± 1.4	0.0033*	2.4 ± 0.3 (S.D. = 1.8)
A. albimanus (Florida Keys, A-3)	928	82.3 ± 1.2		

* Susceptibility greater for *A. albimanus*.

graphically in figure 3, and would indicate that there is no refractory fraction of the Panama *A. albimanus* population or that the refractory portion is very small.

However, in the case of *A. quadrimaculatus* it appears quite certain that there is about a fifth of the population which is refractory. Table 2 indicates that after the infection intensity reaches a level of 20 oöcysts per gut the infected proportion no longer increases, but remains at about 80 per cent.

It can also be noted from the data presented that no *A. quadrimaculatus* lots averaged over 160 oöcysts per infected gut, while, in the Panama *A. albimanus*, averages of over 700 oöcysts per gut were reached. This indicates a definite limitation to infection intensity in *A. quadrimaculatus* where this particular strain of parasite is concerned.

Comparative susceptibility of Florida Keys Anopheles albimanus *and* Anopheles quadrimaculatus. Twenty-seven feedings were undertaken to determine the relative susceptibility of a strain *A. albimanus* from the Florida Keys, and not coindigenous with the strain of malaria used, when fed simultaneously with *A. quadrimaculatus*. The results of these paired feedings are summarized in table 3.

In this comparison, a larger proportion of *A. albimanus* was infected in 23 of 27

paired feedings. Summation of all feedings showed that 82.3 per cent of the Florida Keys *A. albimanus* individuals became infected against 76.8 per cent of the *A. quadrimaculatus*. This difference was found to be highly significant (P = 0.0033).

With regard to infection intensity, *A. albimanus* from the Keys had an average of 2.4 times as many oöcysts as *A. quadrimaculatus* (as compared with 7.2 for the coindigenous Panama *A. albimanus*). Due to the smaller number of feedings it was not possible to determine whether or not a refractory fraction existed in the population (table 2), but if such a fraction does exist it is certainly not large.

Comparative susceptibility of the coindigenous Panama A. albimanus *and the* A. albimanus *from the Florida Keys.* Thirty-two pairs of lots were fed to determine the comparative susceptibility of the two strains of *A. albimanus*. The pattern suggested by the individual susceptibility comparisons of the two strains of *A. quadrimaculatus* was borne out. In 22 of 32 feedings the Panama *albimanus* had a higher proportion infected; in two cases proportions were identical and in the remaining eight there were more infected in the Florida *albimanus* lots. Summation revealed that 86.4

TABLE 4

Summary of 32 paired feedings comparing the susceptibility of Anopheles albimanus *from Panama and* A. albimanus *from the Florida Keys to a Panama strain of* Plasmodium falciparum

MOSQUITO STRAIN	NO. DISSECTED	PER CENT INFECTED	P VALUE	AVERAGE RATIO OF MEAN OÖCYST COUNTS PER INFECTED GUT PANAMA *A. albimanus* FLORIDA KEYS *A. albimanus*
A. albimanus (Panama, A-2)	1,103	86.4 ± 1.1	0.0009*	1.8 ± 0.2 (S.D. = 1.1)
A. albimanus (Florida Keys, A-3)	1,091	81.2 ± 1.2		

* Susceptibility greater for Panama *A. albimanus*.

per cent of the Panama *albimanus* individuals became infected as compared with 81.2 per cent of the Florida strain (table 4). The difference was found to be significant (P = 0.0009).

Study of the relative oöcyst means showed that the Panama strain averaged 1.8 times as many oöcysts per gut as the strain from the Florida Keys.

DISCUSSION

The work of Eyles and Young (1950) demonstrated that *A. quadrimaculatus*, when fed simultaneously with a Panama strain of *A. albimanus*, proved more susceptible to a coindigenous United States strain of *P. falciparum*. The present study indicates that a reciprocal relationship also exists, in that a Panama strain of *A. albimanus* when fed simultaneously with a United States strain of *A. quadrimaculatus*, proved more susceptible to a coindigenous Panama strain of *P. falciparum*. The inclusion of a Florida Keys strain of *A. albimanus*, which was also inferior to the coindigenous Panama strain in susceptibility, lends further support to the contention that the coindigenous vector may be more highly adapted to the malaria strain than the geographically distant vector of the same species.

SUMMARY AND CONCLUSION

When fed simultaneously, two strains of *Anopheles albimanus* proved to be more susceptible than *A. quadrimaculatus* to a Panama strain of *Plasmodium falciparum.* Only about 78 per cent as many *A. quadrimaculatus* became infected as Panamanian *A. albimanus*, and the average oöcyst intensity in the Panama strain was 7.2 times that in *A. quadrimaculatus.* About 93 per cent as many *A. quadrimaculatus* became infected as *A. albimanus* from the Florida Keys, and the average oöcyst intensity in the Florida strain was 2.4 times that in *A. quadrimaculatus.*

In comparing the two strains of *A. albimanus*, about 94 per cent as many of the Florida Keys strain became infected, and the Panama strain averaged 1.8 times as many oöcysts per gut over the Florida strain.

There was probably no refractory portion of the Panama *A. albimanus* population but it appears quite certain that about 20 per cent of the *A. quadrimaculatus* population was refractory.

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A FURTHER REPORT ON THE USE OF CHLORGUANIDE (PALUDRINE) TO SUPPRESS MALARIA PREVALENCE IN SOUTHERN FORMOSAN VILLAGES[1]

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These authors have reported[2] on the use of chlorguanide for one year as a malaria therapeutic and suppressive agent in San Hsing village, near our field station at Ch'ao Chow town in southern Formosa. This study ended in June 1948; but before it was finished we had concluded that the administrative techniques being employed were too complicated for practical use in Formosa. The program reported here was begun in April 1948 and finished in March 1949. It is similar to the first study but simpler in concept, and its administration proved to be less difficult.

STUDY CONDITIONS

Two villages, Ssu Ling and Ssu Ch'un, about six kilometers east of Ch'ao Chow town and four kilometers east of San Hsing village, were used in the study. These villages are about one kilometer apart (Figure 1) and about one and a half kilometers from Lu Liao village. The latter was not protected by any malaria control measure and was used to furnish data for comparison. The drug control villages have populations of about 1,500 persons, while Lu Liao's is about half as large.

The three villages are quite similar in all essential respects. They are discrete and compact groups of houses situated in bamboo groves. Domestic livestock is quartered near the homes. The villages are in close proximity to rice fields which are the site of prolific seasonal propagation of *Anopheles hyrcanus sinensis* while rice is growing, from February to April and from August to October. Irrigation ditches and canals associated with these fields provide breeding places for *Anopheles minimus*, a less important vector than *A. sinensis* in the plains of southern Formosa. All three villages were known to suffer from malaria of moderately severe endemicity.

At first the villagers were eager to co-operate. They had received reports of the beneficial effects of the treatment program at San Hsing village and had sent delegations to solicit extension of this work to their villages. At the outset of the study and for some weeks thereafter this spirit was maintained; but as malaria illness disappeared there was less evident need for the program and less willingness to take the drug. This situation was made worse by the knowledge that San Hsing and other villages had been treated with DDT and without effort on the part of their inhabi-

[1] The studies and observations upon which this paper is based were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation in co-operation with the Taiwan Provincial Malaria Research Institute of the Taiwan Provincial Health Department.

[2] Watson, R. B., Paul, J. H. and Liang, K. C., A report of one year's field trial of chlorguanide (paludrine) as a suppressive and as a therapeutic agent, in southern Taiwan (Formosa), *J. Nat. Mal. Soc.* 9: 25–43, March, 1950.

tants were also nearly free of malaria. Coercion by the village headmen helped for a time to make the villagers queue up for treatment, but after four months it was evident that we would have to terminate the work before the full year of the study plan had passed. We think that a similar experience should be anticipated by anyone who plans to use suppressive therapy under similar conditions.

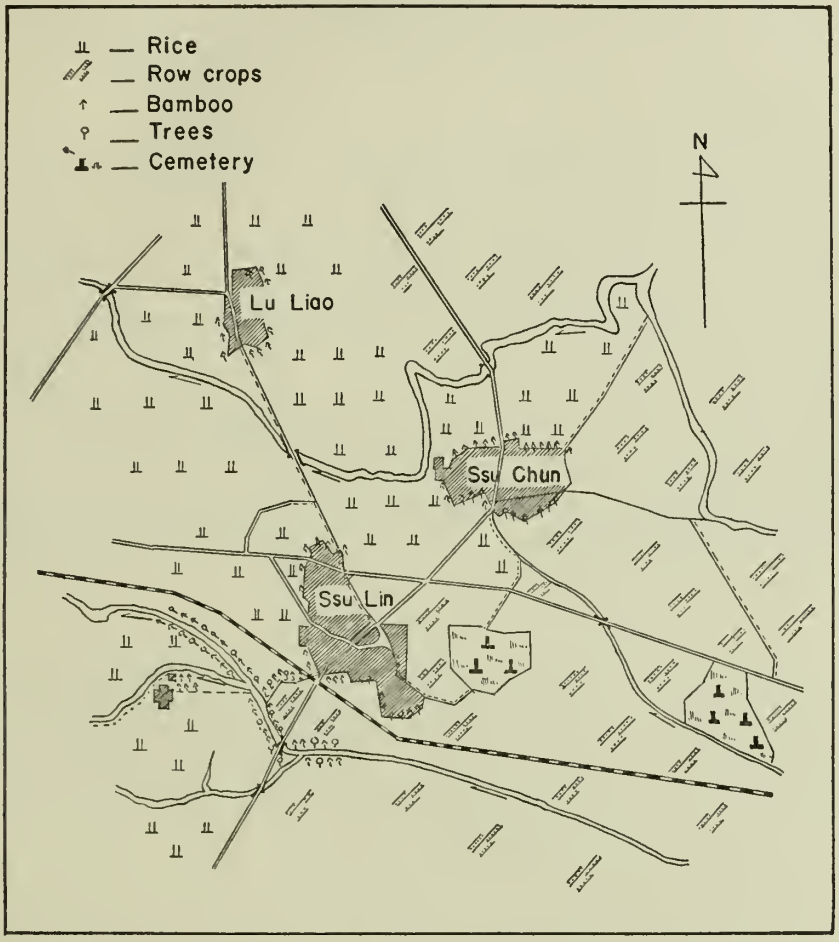

FIG. 1. Map of the Ssu Ling—Ssu Ch'un—Lu Liao area of Ch'ao Chow District, showing the relationship of the villages to each other and to potential mosquito breeding places.

METHODS

Spleen and blood surveys were made of the villagers before the drug was administered, at intervals of about three months thereafter and at the end of the study. In the case of Ssu Ling, these surveys were supplemented by three surveys of school children, the first of which was made in September 1947.

In the original study, all persons with parasitemia received a therapeutic course of chlorguanide and suppressive therapy was given to everyone. In this study, no

attempt was made to find malaria cases as they occurred, or to give chlorguanide routinely to persons reporting sick with malaria. We had re-established the former Japanese government malaria treatment center in Ch'ao Chow and sick persons could avail themselves of this service; some residents of the three villages did so.

Suppressive therapy was administered once every week in Ssu Ling and once every two weeks in Ssu Ch'un, the same doses being given in both villages. The drug was not given to children less than 12 months old, but everyone else was treated. The doses employed were: 1 to 5 years, 0.050 gram; 6 to 15 years, 0.100 gram; 16 years or older, 0.200 gram.

A simple method to speed up administration of the drug gave very good results. Every person to receive chlorguanide had his or her name characters written on a small board. These name boards were left in the care of a boy in each village who knew all the villagers and where they lived. On the day of treatment these boys distributed the boards; they were handed in to the treatment team (a physician and a clerk) when the drug was received. The boys also helped to find persons who did not come in for treatment and sometimes to dispense the drug to these persons, increasingly important duties. This device may not be useful in some places, but it is very helpful in Chinese villages.

The surveys were done on a treatment day, the treatment team being augmented by another physician and clerk and by a technician. The time required each week to dispense chlorguanide was about two hours for each village. No special precautions were taken to insure that the drug was swallowed, as in the former study. The villagers were instructed to swallow the drug with a draught of tea, which was available; we think most of them did so.

RESULTS

General Parasitemia: In Figure 2, which is included for comparison, may be seen seasonal variation of anophelism in the vicinity of Chia Tso during 1949. This place is near Ch'ao Chow and was not protected by malaria control operations of any sort. This figure also shows seasonal variation of parasitism in children attending Chia Tso primary school. We think this figure gives the average picture of seasonal variation of malaria parasitism and anophelism in the plains region of southern Formosa, in the absence of systematic malaria control measures.

Figure 3 shows parasite curves for the three villages under study. It should be noted that chloroguanide administration began at a time when an increase of malaria prevalence was to be expected in the villages. In the villages receiving chlorguanide there was instead a sharp reduction of parasitism. From the initial rates of 13.03 per cent and 10.70 per cent for Ssu Ling and Ssu Ch'un respectively, the rates fell to 1.13 per cent and 1.47 per cent at the second survey. These low rates were maintained at approximately the same levels for the duration of the observations. Rates for three surveys of Ssu Ling primary school are also shown in this figure. They are consistently somewhat higher than the rates for the whole population, as might be expected, but show the same trend of prevalence.

From a practical standpoint, to us the most interesting feature of these data is the fact that there is little difference between the effect obtained by suppressive treatment

with the same doses of chlorguanide at weekly and at biweekly intervals. The rates for Ssu Ch'un village were slightly higher than in Ssu Ling village, but the differences between comparable rates have no statistical significance.

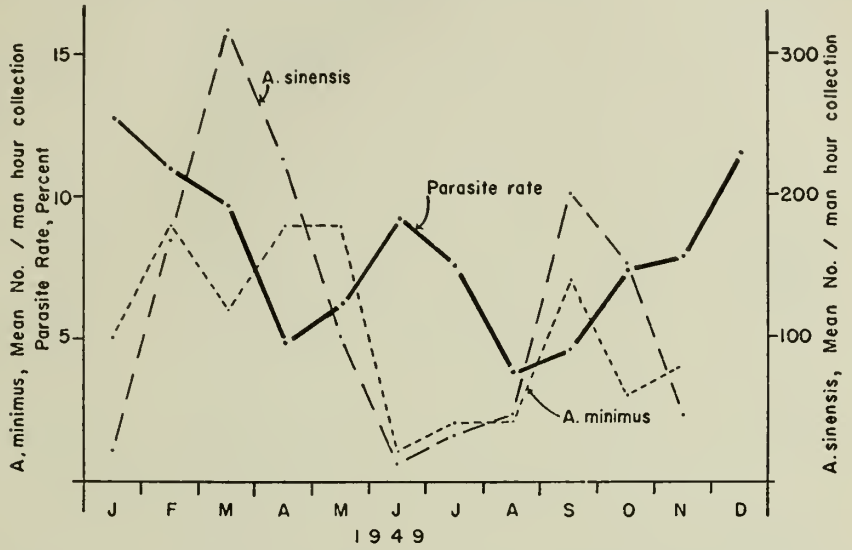

FIG. 2. Variation of malaria parasitism, by months during 1949, in students of Chia Tso primary school. The prevalence of *Anopheles hyrcanus sinensis* and of *Anopheles minimus* for the vicinity of Chia Tso is given for the same period.

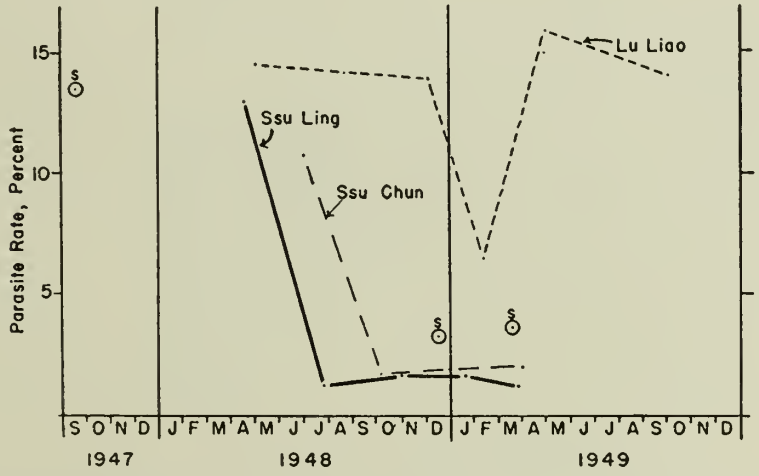

FIG. 3. Variation of malaria parasitemia for the two villages receiving suppressive chlorguanide and for the control village.

In the control village, Lu Liao, the initial rate of 14.73 per cent was followed by similar rates in succeeding surveys, except for the fourth survey, which was done in early February. At this time a decline in malaria prevalence is to be expected, but

TABLE 1

Changes in species parasitemia in the three study villages as determined by blood surveys

	SSU LING (DRUG EVERY WEEK)					SSU CH'UN (DRUG EVERY 2 WEEKS)				LU LIAO (NOT TREATED)					
SURVEY NUMBER	1	2	3	4	5	1	2	3	4	1	2	3	4	5	6
Number examined	1,136	1,066	1,023	912	932	1,421	1,295	992	1,195	387	432	277	239	323	368
Number of infections with															
vivax	43	7	4	3	3	53	5	3	17	8	15	5	2	13	12
falciparum	44	3	8	7	4	38	9	11	4	13	9	16	6	8	28
malariae	58	2	2	4	4	57	5	4	4	35	33	14	8	31	5
mixed inf.	3	0	1	0	0	4	0	0	0	1	1	1	0	0	2
Total positive	148	12	15	14	11	152	19	18	25	57	58	36	16	52	47
Per cent positive	13.03	1.13	1.47	1.54	1.18	10.70	1.47	1.81	2.09	14.73	13.43	13.00	6.70	16.10	12.77

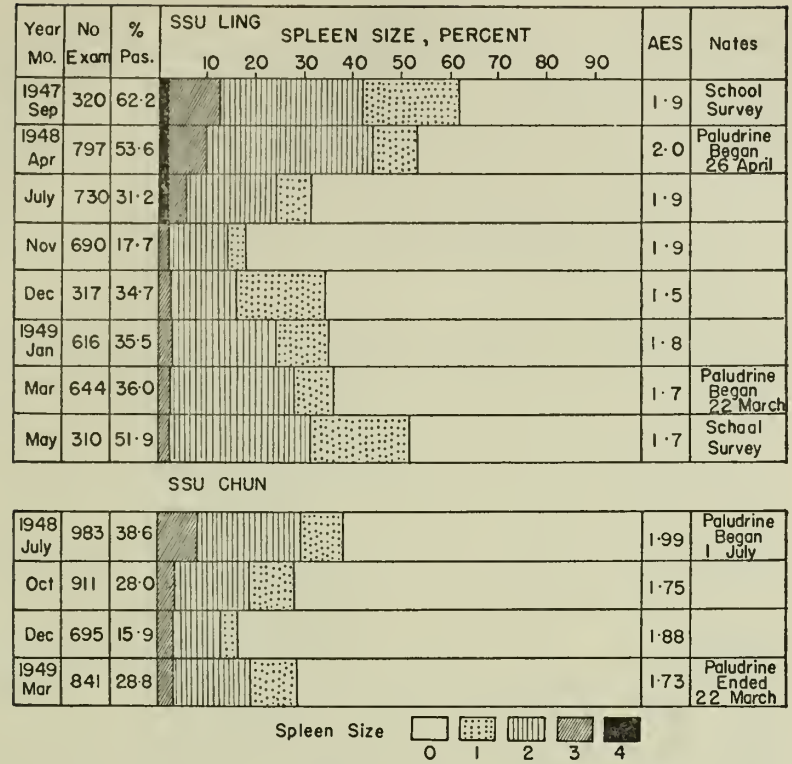

FIG. 4. Variation of spleen rates and sizes in the villages receiving suppressive chlorguanide.

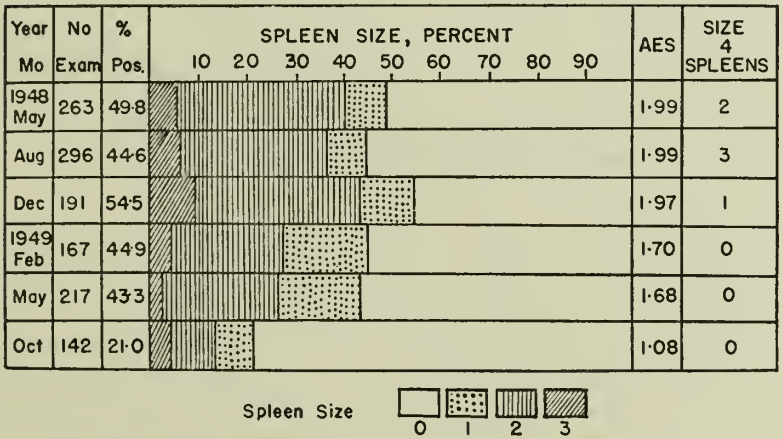

FIG. 5. Variations in spleen rates and sizes in Liu Liao village, which was unprotected by malaria control measures.

a similar decline is also to be expected during midsummer and this did not occur in Lu Liao. We do not have sufficient knowledge of the factors involved to hazard an opinion about the decline in prevalence noted.

Species Parasitism: Table 1 shows numerical data for the blood surveys of the three villages. Perhaps the most striking feature of these data is the fact that infections with *Plasmodium malariae* were most prevalent at the time of the initial surveys, in all three villages. This relative prevalence was maintained in Lu Liao. In Ssu Ling there was no difference between the prevalence of the three species of parasites at the end of the study, but in Ssu Ch'un there was a definite predominance of *Plasmodium vivax* infections. In our earlier study we reported what we supposed to be a tolerance to chlorguanide developed by *P. vivax* when small suppressive doses are given. The longer dosage interval employed in Ssu Ch'un village may have produced a similar effect.

Splenomegaly: Figure 4 shows spleen rates and the relative prevalence of spleens of various sizes as found by surveys of Ssu Ling and Ssu Ch'un. Data for Ssu Ling

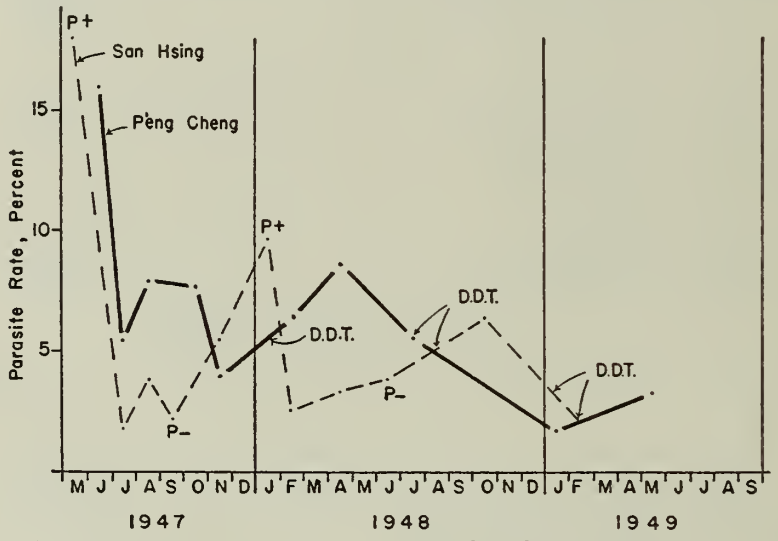

Fig. 6. Changes in parasite rates in San Hsing village, where the use of chlorguanide was followed by DDT residual spray; and for P'eng Cheng village where quinacrine hydrochloride treatment was supplemented and followed by DDT spraying.

village include rates from the school surveys mentioned. The smaller number of spleen examinations than blood examinations for the general surveys is due to the fact that girls over 12 years old were not examined.

In both villages there was a progressive decline in total splenomegaly and in the size of the spleens through the third survey. The fourth survey in both villages gave rates about double the rates for the third survey and the trend in reduction of spleen size was reversed. The rate for the fourth survey in Ssu Ling (34.7 per cent) was maintained in subsequent surveys, though there was a continued increase in the number of size 2 spleens at the expense of size 1 spleens.

In the control village, Lu Liao, there was not much change in spleen rates or spleen sizes during 1948; in 1949 there was a slight reduction of both by the end of the study (Figure 5).

Toxic effects: No toxic effects from the drug were observed.

DISCUSSION

It seems evident from this study that suppressive therapy with chlorguanide in the dosage employed may be expected quickly to reduce malaria parasitism to levels of approximately 1.5 per cent. We were surprised that approximately the same results were obtained with administration of the drug at weekly and at biweekly intervals; also, that the effects obtained with the much simpler administrative technique used gave approximately the same results as in the earlier study. In the latter study it was made certain that all doses of the drug were swallowed and all cases of malaria parasitism were given therapeutic courses of chlorguanide.

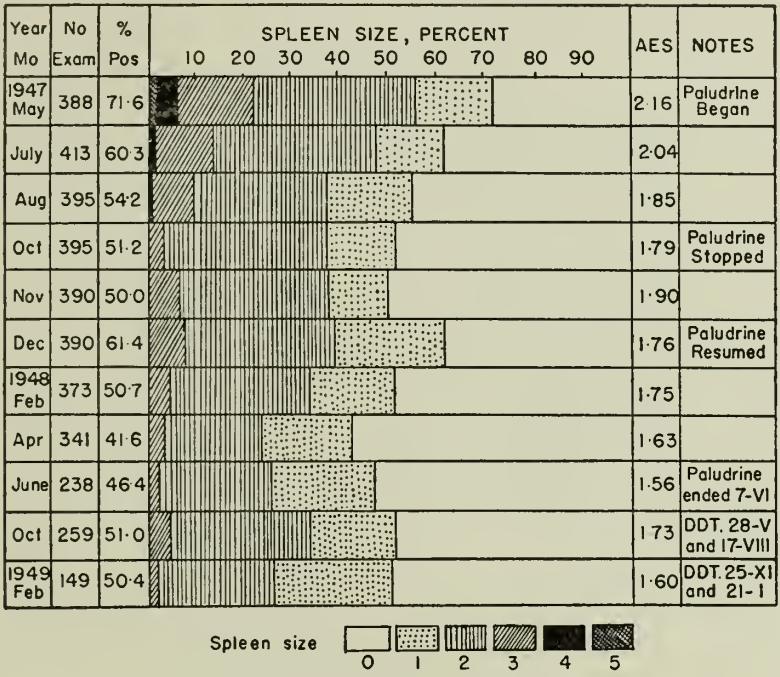

FIG. 7. Changes in spleen rates and sizes in San Hsing village.

From the first study we concluded that while overt malaria is suppressed by chlorguanide, a certain amount of transmission of malaria takes place. Suspension of suppressive therapy in San Hsing village was followed by an immediate rise in parasite rates. From this first study we found also that treatment of malaria cases with quinacrine hydrochloride resulted in a reduction of malaria parasitism to values not much higher than those achieved by chlorguanide suppression and therapy.

Figure 6 shows parasite rates, and Figure 7 and 8 spleen rates, for the original study in San Hsing village and for P'eng Cheng village. In the latter place quinacrine hydrochloride was given systematically to malaria cases applying to a visiting physician for treatment through June 1948. After that time cases were treated at the dispensary in Ch'ao Chow; that is, they had to walk two kilometers to get treatment. The same policy was applied to villagers at San Hsing after suspension of the study in June. Both San Hsing and P'eng Cheng were treated with DDT residual spray

during 1948 and 1949, as shown, "premise spraying" at the average rate of 150 mgms. per square foot being employed. The results of this program, plus treatment of malaria cases at the dispensary, resulted in reduction of malaria prevalence to levels not much different (though a little higher) than did the systematic use of chlorguanide as a suppressive.

At this point we believe that suppressive programs with chlorguanide, or with any other potent antimalarial drug, should be looked upon as an emergency measure, to be replaced as soon as possible by measures directed against anopheline vectors. If chlorguanide suppression is used, we would favor treatment intervals of five days with a maximum dose of 0.30 gram.

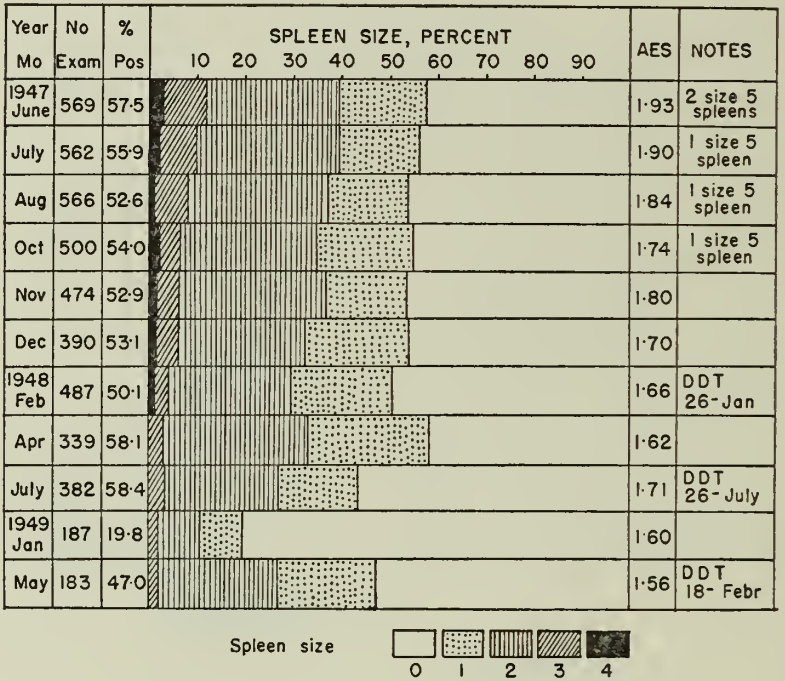

Fig. 8. Changes in spleen rates and sizes in P'eng Cheng village.

Insofar as Formosa is concerned, we believe that the best general approach to malaria control on an island-wide basis is through the use of DDT residual spray, supplemented by the treatment of malaria cases with chlorguanide or another potent drug. We should like to emphasize our conviction of the importance of treating cases of malaria as they occur. It is to be hoped that facilities for early and accurate diagnosis of malaria cases will become generally available, but in most countries where malaria is a serious public health problem today, its treatment probably will have to be upon an empirical basis for some time to come. We believe that treatment centers stocked with the potent and safe antimalarial drugs available today should be established within walking distance of everyone exposed to the hazard of malaria infections, and that the public should be urged to use such facilities.

SUMMARY

In a second study of the use of chlorguanide to suppress malaria prevalence in two Formosan villages, the drug was given in the same dosage once a week in one village and once every two weeks in another, for almost one year. In both villages the administration of the drug was followed by a decline in malaria parasitism to levels of approximately 1.5 per cent, as determined by blood surveys. Spleen rates also declined and the average enlarged spleen size was reduced. In a third village which was unprotected by any malaria control procedure, there was no significant change in parasitism, or in spleen rates and size, during the same period.

In our opinion the new synthetic drugs should usually be reserved for treatment of malaria cases rather than used for suppression programs.

ACKNOWLEDGMENT

The chlorguanide used in this study was supplied gratis by Messrs. Imperial Chemical Industries (China) Ltd., as 100 mgm. and as 10 mgm. tablets. We are particularly grateful to Mr. E. L. L. Wheen for his helpfulness in obtaining the drug for our use.

STUDIES IN HUMAN MALARIA

XXVIII. Observations on the Use of Chlorguanide against the Chesson Strain of *Plasmodium vivax*

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Chlorguanide [N_1-(p-chlorophenyl)-N_5-isopropyl biguanide], also known as paludrine or proguanil, has many properties beside structure which distinguish it from the other major antimalarial drugs. It has an extremely wide margin between the minimal effective and the maximal tolerated dosage. There is evidence that it is active against certain of the fixed tissue stages of plasmodia in man, and it is the only widely used antimalarial to which malarial parasites readily acquire resistance.

In previously reported studies (Lints *et al.*, 1950), we have shown that chlorguanide, like chloroquine, in dosage of 0.3 gm. once weekly will satisfactorily suppress Chesson strain *vivax* malaria, but that even after a year's continuous suppression, parasitemia appears soon after the last dose of drug. We have also shown that the erythrocytic parasites of Chesson strain *Plasmodium vivax* acquire resistance to chlorguanide (Cooper *et al.*, 1950, cf. Seaton and Lourie, 1949) so that a parent strain vulnerable to 1.5 mgm. of chlorguanide per day yielded a resistant substrain not affected by 1,600 mgm. per day.

In the studies to be reported in this paper, several related experiments with chlorguanide will be summarized. The first of these will deal with attempts to determine a minimal effective suppressive dosage of chlorguanide and to determine if this is influenced by the dosage of infective sporozoites. The second will involve attempts to determine if progressively increased suppressive doses in sporozoite-induced infections will result in drug-fastness through action against fixed tissue parasites. The third will involve attempts to cure *vivax* malaria by repeated massive dosage with chlorguanide. The fourth will deal with studies in which chlorguanide and sulfadiazine were given concurrently.

MATERIAL AND METHODS

All of the investigations to be described were carried out in inmate-volunteers at the Federal Correctional Institution, Seagoville, Texas, and involved previously outlined procedures (Coatney *et al.*, 1948). The volunteers were infected with the Chesson strain of *P. vivax* by the bites of insectary-reared *Anopheles quadrimaculatus* mosquitoes, which had fed upon suitable gametocyte carriers two weeks earlier. After feeding, usually upon three volunteers in turn, each mosquito was dissected and the number of sporozoites in its salivary glands graded on a scale of 0 to 4+.

Volunteers remained under close observation for 18 months, during which period blood smears were taken at least once weekly. During acute attacks of malaria, the

men were hospitalized, blood smears were made daily and temperature recordings were made at least every four hours.

Chlorguanide was given as the monohydrochloride (87 per cent base), in tablets containing 100 mgm. of the salt.[1] Dosages refer to the salt content unless otherwise stated. Sulfadiazine was given in tablets of 0.5 gm. each.

Concentrations of chlorguanide in blood plasma were estimated by the hydrolytic method of Spinks and Tottey (1946), while sulfadiazine concentrations in whole blood were estimated by the method of Bratton and Marshall (1939).

LONG-TERM SUPPRESSION WITH GRADUATED DOSAGES OF CHLORGUANIDE

Chlorguanide in dosage of 300 mgm. of base once weekly has been found to be protective against Chesson strain *vivax* malaria induced by the bites of ten mosquitoes, but malaria appeared 19 to 24 days after the drug was stopped at the end of six or 12 months (Lints *et al.*, 1950). Packer (1947) found that doses as low as 87 mgm. of base per week effectively suppressed the McCoy strain of *P. vivax* in two patients bitten on three occasions over a period of one week.

In an attempt to define the minimal suppressive dosage more accurately, particularly with reference to heavy doses of sporozoites, the experiment to be described was carried out. As an important corollary it was possible to determine the chlorguanide susceptibility of the parasites that appeared after suppression ceased.

Experiment. Eighteen volunteers were given chlorguanide in dosages ranging from 50 to 200 mgm. once weekly as shown in Table 1, the first dose being given on the third day before the day of bites, the second dose on the fourth day after the day of bites and additional doses once weekly thereafter for the indicated periods.

On day 0 (10 June 1948) the men were bitten by mosquitoes; 30 infected mosquitoes were allowed to feed upon each of the first nine men and three infected mosquitoes upon each of the last nine men.

Blood smears were taken at least every other day. There was no demonstrable parasitemia or fever in any of the treated men until after the drug was discontinued.

After six weeks, drug was discontinued in the first volunteer in each set of three; malaria appeared within 13 to 22 days. In the remaining two men in each set drug was continued for one year, the last doses being given on day 361. Malaria appeared in all but one man (S-188, who remained negative until discharged 184 days later). In three of the individuals bitten by only three mosquitoes the latent intervals from last dose of drug to patency were quite prolonged (61, 82 and 141 days) when compared with the latent period of two to four weeks usually seen after chlorguanide administration. The delayed infections were not in bite-mates, i.e. men bitten by identical mosquitoes.

Each malarial attack was treated with chlorguanide in single dose of 100 mgm. of salt. In all instances parasite clearance and amelioration of fever were as rapid as expected for chlorguanide. Thus, in the six individuals who had been given chlorguanide for 39 days, the time required for parasite clearance by 100 mgm. of chlor-

[1] Supplied through the kindness of E. I. du Pont de Nemours & Co.

guanide, was two to eight days (median 5.5) and in 11 individuals who had been given chlorguanide for 361 days clearance time ranged from two to nine days (median 6.0 days). Fever was relieved by therapy and relapses did not occur until 13 to 33 days later. With treatment of successive attacks, using 100 mgm. of chlorguanide, there was the eventual appearance of resistant parasites in some of the subjects.

Comment. Even when 30 heavily-infected mosquitoes provided the inoculum, 50 mgm. of chlorguanide hydrochloride (44 mgm. of base) once weekly afforded satis-

TABLE 1

Long term suppression of Chesson strain vivax *malaria with chlorguanide*

VOLUNTEER NUMBER	INFECTIVE INOCULUM		CHLORGUANIDE HYDROCHLORIDE MGM. ONCE WEEKLY BEGINNING 3 DAYS BEFORE BITES	ADMINISTRATION STOPPED (DAY AFTER BITES)	DAYS FROM LAST DOSE OF DRUG TO FIRST PATENT PARASITEMIA
	Number of mosquitoes	Sum of pluses*			
S-178	30	113	200	39	18
S-179	30	110	200	361	16
S-180	30	111	200	361	22
S-181	30	112	100	39	14
S-182	30	110	100	361	18
S-183	30	115	100	361	18
S-184	30	111	50	39	17
S-185	30	114	50	361	14
S-186	30	113	50	361	28
S-187	3	12	200	39	22
S-188	3	11	200	361	—
S-189	3	12	200	361	18
S-190	3	12	100	39	17
S-191	3	11	100	361	61
S-192	3	11	100	361	14
S-193	3	11	50	39	13
S-194	3	11	50	361	82
S-195	3	12	50	361	141

* Sum of the individual sporozoite densities (1+ to 4+) in the mosquitoes which bit each volunteer.

factory protection, so that the minimal suppressive regimen under these conditions of exposure was not defined.

Small inocula of sporozoites, resulting from three mosquito bites, were able in all but one instance to produce infections which survived 12 months of suppression.

The results with chlorguanide differ from those we have observed with chloroquine (unpublished observations) where it was found that with 30-mosquito inoculations, and 0.1 gm. of chloroquine per week, transitory breakthroughs occurred at 10 to 12 days after mosquito bites. These were controlled, however, by the regularly sched-

uled weekly dose of chloroquine, with no subsequent breakthroughs. The probable explanation is that chloroquine does not interfere with pre-erythrocytic development, and that the simultaneous introduction of many red cell-invading forms into the circulation about days 8 to 12 is sufficient to produce a brief febrile response and, occasionally, enough parasites to be detected by thick smear. With chlorguanide, maturation of pre-erythrocytic stages presumably does not occur, even at the low weekly dosage employed in these studies.

Prolonged suppression did not lead to drug-resistance. There were no breakthroughs late in the period of suppression, and the erythrocytic parasites which appeared two or more weeks after the last dose of drug were normally susceptible to chlorguanide.

The use of low doses of chlorguanide (100 mgm.) for therapy precluded study of long-term relapse patterns, inasmuch as this dosage permitted survival of a few erythrocytic parasites. These parasites eventually developed drug-resistance so that the actual picture of repeated relapses from fixed-tissue sites was obscured.

ATTEMPTS TO INDUCE DRUG-FASTNESS BY PROGRESSIVELY-INCREASED DOSAGES OF CHLORGUANIDE IN SPOROZOITE-INDUCED INFECTIONS

Although the repeated administration of small doses of chlorguanide over long periods of time did not lead to drug-resistance, it was felt that this did not completely rule out the possibility that resistance might be acquired through action upon fixed-tissue parasites. A more critical test would be provided if the parasites, from the moment of introduction in the body, were exposed to minute concentrations of the drug, which could then be progressively increased. This would more nearly duplicate the conditions under which resistance in erythrocytic parasites had been developed (Cooper *et al.*, 1950). Such an experiment was designed, in which action upon erythrocytic parasites could be kept at a minimum, and could be ruled out as a possible factor in the final appraisal.

Experiment. Two volunteers (S-258 and S-261) bitten by ten infected mosquitoes each on 1 December 1949 were given chlorguanide beginning on the day of exposure. Dosage for the first week was 1.5 mgm. per day, that for the second week was 3.125 mgm. per day, and the dosage was doubled each week until 10 weeks after exposure, when 800 mgm. per day were being given. At no time during this period did parasitemia or fever occur.

On the morning following the last dose of chlorguanide 200 ml. subinoculations were made from S-258 and S-261 to recipients who had no history of malaria. This was to determine whether or not viable, chlorguanide-resistant, erythrocytic parasites were circulating. The recipients did not develop malaria.

Following the subinoculations, S-258 and S-261 were treated with quinine sulfate, 2 gm. per day for seven days, to eradicate any circulating erythrocytic forms.

In both men, patent parasitemia appeared 13 days after the last doses of quinine. The parasites in these attacks, which presumably had come from fixed-tissue parasites exposed to ten weeks of progressively increased chlorguanide, were tested for their sensitivity to chlorguanide in two ways:

(1) Subinoculations of 10 ml. of blood were made from S-258 and from S-261 to

two recipients each, as shown in figure 1. The number of parasites transferred ranged from 2.8 to 3.3 $\times$ 10^6. The recipients were treated with dosages of chlorguanide just above the amounts which were known to be necessary to eradicate erythrocytic parasites in blood-induced infections with the parent strain (Cooper *et al.*, 1950).

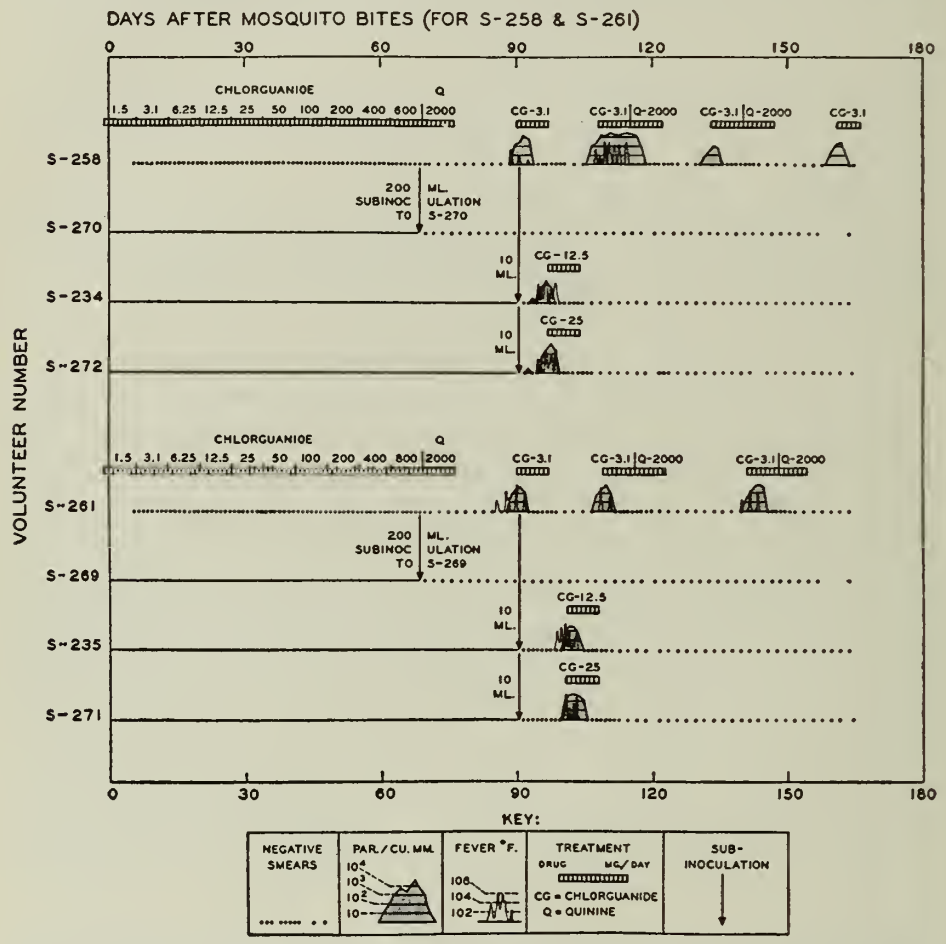

FIG. 1. Courses of therapy and subinoculations in 8 volunteers used in attempts to induce drug-resistance by progressively increased dosages of chlorguanide in sporozoite-induced Chesson strain vivax malaria.

These dosages (12.5 and 25 mgm. per day for seven days) were curative in all instances.

(2) S-258 and S-261 were each treated with chlorguanide in small dosage, 3.125 mgm. per day for seven days, twice the minimal dosage necessary for temporary clearance of parasitemia in the parent strain (Cooper *et al.*, 1950). Parasitemia and fever were cleared promptly. Relapses occurred 10 and 11 days later.

Comment. The foregoing results indicate that the erythrocytic parasites of the

first attack following step-like progression of chlorguanide dosage from 1.5 mgm. per day to 800 mgm. per day, over a period of ten weeks, are as susceptible to chlorguanide as those of the parent strain. In the preceding section it was shown that the prolonged weekly administration of chlorguanide, adequate for complete suppression of erythrocytic parasites, did not lead to detectable drug-fastness. From these data it can be inferred that the fixed tissue parasites of *P. vivax* do not share with the red blood cell parasites the ability for rapid acquisition of drug fastness. This bears out the prediction of Rollo, Williamson and Lourie (1948) who, working with *P. gallinaceum*, found that prolonged treatment of latent infections with paludrine did not result in drug-resistance.

THERAPY OF CHESSON *VIVAX* MALARIA WITH REPEATED COURSES OF CHLORGUANIDE

The inability of chlorguanide to cure *vivax* malaria, in spite of the strong evidence for its activity against exo-erythrocytic parasites, has puzzled investigators since first demonstrated by Fairley (1946). It has been postulated that the drug is a plasmodistatic rather than a plasmodicidal agent, so that the pre-erythrocytic stages revive and proceed with their development after treatment. Another possibility, of course, is that the pre-erythrocytic stages are actually destroyed but that some other less actively metabolizing stage of the parasite survives to initiate relapses.

The results of treatment of several patients with maximum tolerated dosages of chlorguanide, given repeatedly, at the time of each relapse, are presented as evidence that any partially curative effect must be of a very low order.

Experiment. Four volunteers (S-108 to S-111), inoculated with Chesson strain *vivax* malaria on 9 July 1947 by the bites of ten mosquitoes with moderately heavy infection, were treated with chlorguanide in dosage of 1.0 gram per day for 14 days, at the time of their primary attacks and at each relapse thereafter. Parallel cases, infected by the same lot of mosquitoes, were treated with chloroquine in dosage of 800 mgm. per day on the first day and 400 mgm. per day for 13 days thereafter, i.e. 6.0 grams in 14 days. The pattern of relapses in the two groups can be seen in Table 2 and Figure 2.

In terms of total numbers of attacks and intervals between successive attacks, the chlorguanide regimen was inferior. There was no evidence, even with massive dosages, that the total life of the underlying fixed tissue stages was shortened. The final relapses in the chlorguanide-treated men began on days 100, 148, 314 and 403, whereas in the chloroquine-treated patients, the final relapses began on days 289, 353, 367 and 367. The chlorguanide-treated patients experienced three to six attacks each (average 4.8) while the chloroquine-treated patients experienced three to four attacks each (average 3.8).

Comparative data on parasite clearance and alleviation of fever in the two groups were obtained, based on the first three attacks in each volunteer: chlorguanide cleared parasitemia in three to seven days (mean 5 days, median 5.5 days); fever occurred after the beginning of treatment in nine of 12 cases, the last fever of 101°F. being recorded eight to 60 hours (mean 33 hours, median 32 hours) after first dose of drug. Chloroquine cleared parasites in one to three days (mean 2.1, median 2.0 days); in six of 11 cases fever occurred after treatment began, the last fever in these

TABLE 2

Comparison of relapses in volunteers treated with chlorguanide and in parallel cases treated with chloroquine; all men were bitten by ten infected mosquitoes from the same lot and observed for 546 days

VOLUNTEER NUMBER	SET NUMBER*	INFECTIVE INOCULUM (SUM OF PLUSES†)	REGIMEN USED IN THERAPY OF EACH ATTACK	TOTAL NUMBER OF ATTACKS	DAYS FROM END OF TREATMENT UNTIL RELAPSE, BETWEEN ATTACKS:							ONSET OF LAST ATTACK (DAY AFTER BITES)
					1&2	2&3	3&4	4&5	5&6	6&7	7&8	
S-108	3	26	Chlorguanide HCl,	3	25	30	—	—	—	—	—	100
S-109	4	39	1.0 gram per day	6	23	25	29	28	112	—	—	314
S-110	5	29	for 14 days.	6	25	26	28	70	157	—	—	403
S-111	1	29		4	21	25	40	—	—	—	—	148
S-112	2	32	Chloroquine, 0.8	3	81	61	—	—	—	—	—	289
S-113	3	26	gram of base on	4	74	79	136	—	—	—	—	353
S-114	4	40	first day; 0.4	4	87	86	129	—	—	—	—	367
S-115	5	29	gram per day for 14 days.	4	87	80	135	—	—	—	—	367

* Volunteers with the same set numbers were bitten by the same mosquitoes.

† Sum of the individual sporozoite densities (1+ to 4+) in the mosquitoes which bit each volunteer.

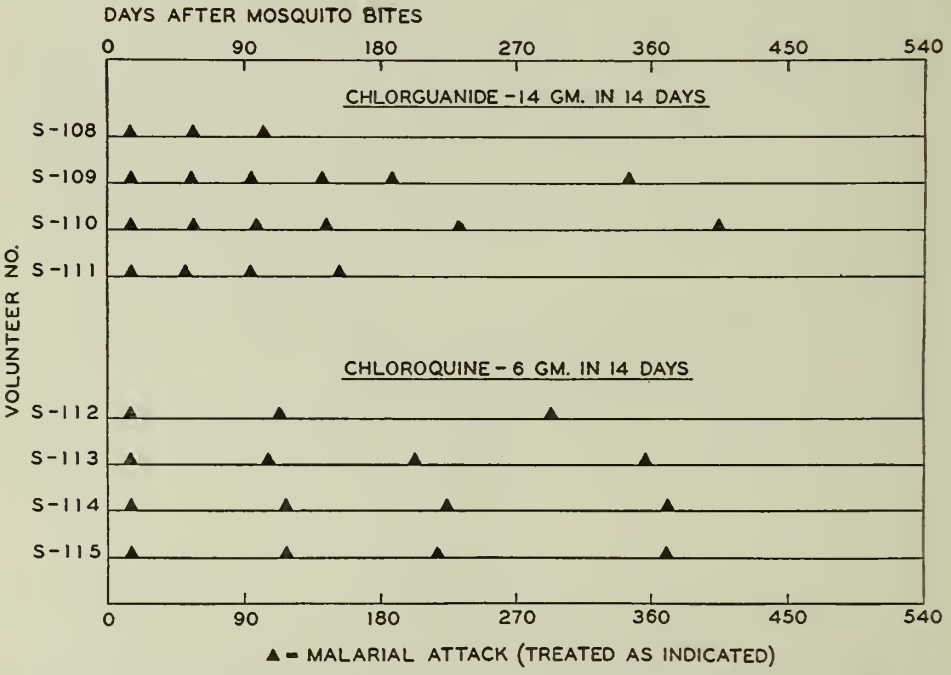

FIG. 2. Pattern of attacks in volunteers S-108 through S-111, treated at each attack with large doses of chlorguanide, compared with the pattern in volunteers S-112 through S-115, treated with chloroquine. Presentation of attacks is diagrammatic.

six occurring at eight to 40 hours (mean 27 hours, median 30 hours) after the first dose of drug.

Comment. The fact that relapses continue to occur after repeated high dosage with chlorguanide and that such dosage does not materially reduce the total duration of the infection favors the view that the drug is failing to reduce the number of fixed tissue parasites presumably responsible for relapses. This is not incompatible with the view that chlorguanide inhibits or destroys the actively maturing tissue stages which immediately precede invasion of red blood cells. At the same time, there is no proof that the stages which survive to produce relapses are actual descendants of forms such as those described by Shortt *et al.*, (1948). The pre-erythrocytic stages which have been demonstrated in man and monkey may be present only just prior

TABLE 3

The attempted cure of Chesson strain vivax *malaria with chlorguanide and sulfadiazine in combination. All volunteers were infected on the same date by the bites of ten infected mosquitoes*

VOLUNTEER NUMBERS	REGIMEN	TYPE OF ATTACK	NO. RELAPSES/ NO. TREATED	DAYS FROM END OF THERAPY TO RELAPSE
S-130 thru S-134	Chlorguanide, 1.0 gram + sulfadiazine 4 grams per day for 14 days.	Primary Second	5/5 5/5	25, 30, 25, 29, 23 37, 165, 71, 160, 24
S-135, 136, 137	Sulfadiazine, 4 grams per day for 14 days.	Primary Second	3/3* 3/3*	21, 19, 19* 26, 43, 27*
S-138 & 139	Chlorguanide, 1.0 gram per day for 14 days.	Primary Second	2/2 2/2	21, 33 22, 66
S-140 & 141	Quinine, 2.0 grams per day for 14 days.	Primary Second	2/2 2/2	7, 35 10, 22
S-142 & 143	Pentaquine, 0.06 gram + quinine 2.0 grams per day for 14 days.	Primary Second	1/2 0/1	37, — —

* Quinine, 2.0 grams daily for 7 days was necessary to clear erythrocytic parasites. Intervals to relapse were calculated from the last dose of sulfadiazine.

to the period of red cell invasion, and some yet unrecognized precursor may be the resistant stage responsible for long latency. (cf. Shute, 1946).

COMBINATION OF CHLORGUANIDE AND SULFADIAZINE

Greenberg *et al.*, (1949) reported a high degree of synergism between chlorguanide and sulfadiazine, against both erythrocytic and exo-erythrocytic forms of *P. gallinaceum* in the chick. The concurrent administration of $\frac{1}{4}$ minimal effective dosage of chlorguanide and $\frac{1}{32}$ minimal effective dosage of sulfadiazine was effective. These results justified prophylactic and therapeutic trials of combinations in man.

Experiments. For the attempted cure of *vivax* malaria each of 14 volunteers was experimentally infected by the bites of ten mosquitoes on 16 December 1947. They developed primary attacks 9 to 11 days later. The first five were treated with sulfadiazine, 4 gm. per day (1 gm. every 6 hours) plus 1.0 gm. of chlorguanide per day (0.25 gm. every 6 hours) for 14 days. The remaining nine men were utilized for sul-

fadiazine, chlorguanide, quinine and pentaquine-quinine controls, as shown in Table 3. Sulfadiazine did not clear erythrocytic parasitemia, so that followup therapy with 2 gm. of quinine sulfate per day for 7 days was necessary. Relapses occurred in all subjects treated with the chlorguanide-sulfadiazine combination, 23 to 30 days after therapy, which corresponded to figures for chlorguanide alone. Four of the five patients given combined therapy continued to have relapses after three courses.

TABLE 4

The attempted prophylaxis of Chesson strain vivax *malaria with sulfadiazine and chlorguanide, alone and in combination*

REGIMEN	VOLUNTEER NUMBER	SET NUMBER*	INFECTIVE INOCULUM		MEAN CONCENTRATIONS		FIRST PATENT PARASITEMIA (DAYS AFTER BITES)	FIRST FEVER 101°F. (DAYS AFTER BITES)
			Number of mosquitoes	Sum of pluses†	Sulfadiazine mg./100 cc.	Chlorguanide γ/liter		
Sulfadiazine, 4 grams per day + chlorguanide HCl 1 gram per day for 2 days before, day of, and 6 days after bites (2–1–6)	S-144	1	10	32	8.5	667	66	69
	S-145	2	10	32	7.1	496	69	68
	S-146	3	10	32	8.4	811	74	75
	S-147	4	10	37	7.6	650	37	38
	S-148	5	10	35	9.3	838	36	38
Sulfadiazine, 4 grams per day (2–1–6)	S-149	1	10	32	9.1	—	14	13
	S-150	6	10	37	7.8	—	12	12
	S-151	2	10	32	7.3	—	12	12
Chlorguanide HCl, 1 gram per day (2–1–6)	S-152	1	10	32	—	695	28	29
	S-153	4	10	36	—	657	34	35
	S-154	3	10	36	—	629	54	57
Controls, no drug	S-155	5	10	32	—	—	12	11
	S-156	4	10	36	—	—	10	11
	S-157	6	10	37	—	—	12	10
	S-158	2	10	32	—	—	12	13
	S-159	3	10	30	—	—	14	13

* Volunteers with the same set numbers were bitten by the same mosquitoes.

† Sum of the individual sporozoite densities (1+ to 4+) in the mosquitoes which bit each volunteer.

For attempted prophylaxis 16 volunteers were used (Table 4). Five were given 4 gm. of sulfadiazine per day plus 2.0 gm. of chlorguanide per day for two days before, on the day of, and for six days after exposure to ten infected mosquitoes. Three men were given sulfadiazine alone for the same period, three were given chlorguanide alone and five served as untreated controls. All of the 16 volunteers developed malaria, the controls and sulfadiazine-treated subjects first showed parasitemia ten to 14 days after exposure, those given chlorguanide at 28 to 54 days, and those given the combination of chlorguanide and sulfadiazine at 36 to 74 days.

Comment. Combined chlorguanide and sulfadiazine showed no antimalarial effects against *vivax* malaria beyond those expected of chlorguanide alone.

SUMMARY AND CONCLUSIONS

Studies in experimentally-infected prisoner-volunteers are reported which show that sporozoite-induced Chesson strain *vivax* malaria, whether produced by the bites of 30 or three heavily infected mosquitoes, was successfully suppressed for one year by doses of 200, 100 or 50 mgm. of chlorguanide hydrochloride once weekly. Malaria appeared after suppression in all but one of 18 subjects. The erythrocytic parasites of the delayed attacks were susceptible to single 100 mgm. doses of chlorguanide. Such low dosages are not recommended in practice because of the likelihood of natural and induced drug-resistance.

The administration of progressively increased doses of chlorguanide, starting on the day of mosquito bites with 1.5 mgm. per day for one week and ending with 800 mgm. per day during the 10th week after bites, did not result in drug-resistance in the parasites that appeared after suppression was discontinued. It was concluded that the erythrocytic forms of *P. vivax* alone have the potentiality for ready acquisition of chlorguanide-resistance. The ability of the erythrocytic forms to become resistant is so great, however, that it would seem inevitable that resistance would develop at times under natural conditions.

The failure of large doses of chlorguanide to cure *vivax* malaria was confirmed. This was true even when successive attacks in patients were treated with one gram of chlorguanide per day for 14 days. Both in terms of rapidity of action and total number of attacks per man, chlorguanide was slightly inferior to chloroquine, which was used in parallel cases.

No significant degree of synergism, either in prophylactic or therapeutic action, was observed when chlorguanide and sulfadiazine were given concurrently.

SUMARIO Y CONCLUSIONES

Estudios llevados a cabo con prisioneros voluntarios revelaron que las infecciones inducidas con esporozoitos de la cepa Chesson de malaria a *vivax* producidas indiferentemente por la picada de 30 6 de 3 mosquitos altamente infectados fué suprimida por un año mediante dosis de 200, 100 6 50 mgm. de clorhidrato de cloroguanida. Tales dosis no se recomiendan en la práctica por la probablidad de que se desarrolle una resistencia natural o inducida a la droga.

La administración de dosis progresivamente crecientes de cloroguanida, empezando el día de la picada de los mosquitos con 1.5 mgm. diarios por una semana y terminando con 1.5 mgm. diarios por una semana y terminando con 800 mgm. diarios durante la décima semana después de la picada no produjo resistencia a la droga en los parásitos que aparecieron después que cesó la supresión. Se concluyó que solamente las formas eritrocíticas de *Plasmodium vivax*, tienen capacidad de adquirir rápidamente resistencia a la cloroguanida. Sin embargo, la habilidad de las formas eritrocíticas a hacerse resistentes es tan grande, que parece inevitable que se desarrolle a veces resistencia bajo condiciones naturales.

La falla de grandes dosis de cloroguanida para curar la malaria a *vivax* fué confirmada. Esto resultó cierto aún cuando ataques sucesivos fueron tratados con 1.0 gm. diario de cloroguanida durante 14 días. Tanto en rapidez de acción como en

número total de ataques por hombre la cloroguanida fué ligeramente inferior que la cloroquina la cual fué usada en casos paralelos.

No se observó ningún grado de sinergismo ni en su acción profiláctica ni en su cación terapéutica, mediante el uso conjunto de la cloroguanida y la sulfadiacina.

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STUDIES IN HUMAN MALARIA

XXIX. Trials of Aureomycin, Chloramphenicol, Penicillin, and Dihydrostreptomycin against the Chesson Strain of *Plasmodium vivax*

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The antibiotics have been conspicuous in their ineffectiveness against malarial parasites. There have been a few exceptions, however, in experimental infections. Taliaferro, Coulston and Silverman (1944) showed intravenously-administered tyrothrycin to be active against *Plasmodium gallinaceum*. Seeler, Malanga and Pierson (1945) reported that, while streptomycin was ineffective as a suppressant of *P. cathemerium*, *P. gallinaceum* and *P. lophurae*, it had a slight effect upon sporozoite-induced *gallinaceum* malaria. Gramicidin has been reported active against *P. gallinaceum* in the chick (Wiselogle, 1946).

Although Deshmukh (1947) reported lowered relapse rates in *vivax* malaria when penicillin was given in combination with quinine and mepacrine, the studies cannot be regarded as adequately controlled, and they have not been confirmed. Penicillin is not active in the avian malarias (Wiselogle, 1946), or against the erythrocytic parasites of *P. vivax* (Hindle *et al.*, 1945).

When it was found in this laboratory that aureomycin was effective against *P. gallinaceum* in the chick (Coatney *et al.*, 1949), clinical trials were started. Volunteers, bitten by mosquitoes infected with the Chesson strain of *P. vivax*, were given the drug prophylactically and therapeutically (Cooper *et al.*, 1949). A dosage of 8 gm. per day for one week delayed acute attacks for two to three weeks beyond those in controls and was slightly effective against erythrocytic parasites. In order to confirm, if possible, this antimalarial activity which was of theoretical interest, an additional experiment was carried out using smaller dosages of aureomycin. Parallel cases were given chloramphenicol (chloromycetin), dihydrostreptomycin, and penicillin. This report will summarize the second set of experiments.

MATERIALS AND METHODS

The general methods were those that have been followed in all chemotherapeutic trials at the Federal Correctional Institution at Seagoville, Texas, as previously described by Coatney *et al.*, (1948). Each volunteer was bitten by 10 mosquitoes from a heavily-infected lot; after feeding, each mosquito was dissected and the number of sporozoites in its salivary glands graded on a scale of 0 to ++++.

Volunteers were followed closely after inoculation, with blood smears made daily from the sixth day through the first attack, and at least every other day thereafter through six months of observation, at which time the experiments were concluded.

Each malarial attack was interrupted promptly with treatment begun on the third to the fifth day of patent parasitemia.

Aureomycin hydrochloride, in 250 mgm. capsules, was furnished through the kindness of the Lederle Laboratories Division, American Cyanamid Company. Chloramphenicol (Chloromycetin) was supplied by Parke, Davis & Co. Penicillin was given in the form of combined procaine penicillin G plus buffered crystalline sodium penicillin, in ratio of 3:1, each 5 ml. of combination containing 4,000,000 units. Dihydrostreptomycin was given as the hydrochloride, while chloroquine was given as the diphosphate (62 per cent base).

TABLE 1

Trial of aureomycin, chloramphenicol, penicillin, and dihydrostreptomycin as prophylactic and curative agents against sporozoite-induced Chesson strain vivax *malaria*

SET NO.	VOL. NO.	INFECTED MOSQUITOES PER SUBJECT ON DAY 0		PROTECTIVE REGIMEN	PRE-PATENT PERIOD	INCUBATION PERIOD 101°F	NUMBER ATTACKS THROUGH DAY 180	NUMBER OF DAYS FROM CHLOROQUINE TREATMENT TO RELAPSE BETWEEN ATTACKS			
		No.	Sum of pluses					1&2	2&3	3&4	4&5
					days	*days*					
3	S-263	10	31	Aureomycin	28	27	4	32*	37	35	—
4	S-266	10	35		29	29	3	36*	36	—	—
3	S-264	10	29	Chloramphenicol	22	23	3	61*	35	—	—
4	S-267	10	35		18	18	4	28*	37	33	—
1	S-259	10	36	Penicillin	13	13	4	32	41	47	—
2	S-262	10	35		12	12	4	30*	42	42	—
1	S-257	10	37	Dihydrostreptomycin	12	11	5	28*	31	41	25
2	S-260	10	36		11	12	4	34*	46	69	—
3	S-265	10	28	None	14	14	4	36	51	36	—
4	S-268	10	35		11	11	4	27	47	49	—

* Chloroquine therapy (0.6 gm. of base) of the primary attacks in these men was followed by the same antibiotic regimen used for attempted prophylaxis.

EXPERIMENTS AND RESULTS

Attempted prophylaxis. Ten volunteers were used for the prophylactic trials. Administration of drug was begun on the morning of the bite-day, before exposure to 10 infected mosquitoes, and for six days after the day of bites. Each of the following regimens was tested in two volunteers, two men serving as controls:

1. Aureomycin hydrochloride 4 gm. per day (0.5 gm. orally every 3 hours);
2. Chloramphenicol, 4 gm. per day (0.5 gm. orally every 3 hours);
3. Penicillin G (rapid + repository) 1,000,000 units per day (500,000 units intramuscularly every 12 hours);
4. Dihydrostreptomycin hydrochloride, 2 gm. per day (1 gm. intramuscularly every 12 hours).

As shown in table 1, the controls and the volunteers given penicillin or dihydrostreptomycin developed patent malaria 11 to 14 days after inoculation. There was a delay until days 18 and 22 in the appearance of parasites in the two men given chloramphenicol, and a considerably greater delay, 28 and 29 days, respectively, in the two given aureomycin.

Attempted cure. In all ten acute attacks erythrocytic parasitemia was controlled by the administration of chloroquine (1.0 gm. of diphosphate = 0.6 gm. of base, in two divided doses 6 hours apart). This was followed in each case by the drug regimen that had been used in attempted protection of the patient, i.e. volunteers S-263 and S-266 were given aureomycin, 4 gm. per day for 7 days, S-264 and S-267 were given chloramphenicol 4 gm. per day, etc. The only exception was S-259, who developed severe urticaria at the time of his second attack, which was interpreted as possibly representing a reaction to the earlier course of penicillin. In each volunteer relapse occurred, the intervals from chloroquine therapy to relapse not differing significantly from those in the controls treated with chloroquine alone. Subsequent attacks in all men were interrupted with chloroquine. At the end of six months there was no evidence that the courses of antibiotics had reduced the relapse potential of any case.

SUMMARY AND CONCLUSIONS

Aureomycin, chloramphenicol, penicillin, and dihydrostreptomycin were tested as prophylactic and curvative agents in a small series of closely controlled sporozoite-induced Chesson strain *P. vivax* infections. The experiments were primarily designed to test for activity against fixed-tissue parasites, and chloroquine was used to eliminate erythrocytic parasites in the curative trials.

Aureomycin in dosage of 4 gm. per day for 6 days after exposure produced significant delay in the appearance of acute attacks, confirming earlier results when 8 gm. per day were given (Cooper *et al.*, 1949). Thus aureomycin either inhibits the development of pre-erythrocytic parasites or, by residual activity, interferes with the survival and multiplication of early erythrocytic forms. Aureomycin was not curative and did not prolong intervals to relapse beyond those achieved by chloroquine alone.

Chloramphenicol in dosage of 4 gm. per day for 6 days also delayed acute attacks slightly, 18 and 22 days as compared with 11 to 14 days in controls. Inasmuch as the range of prepatent periods in 96 consecutive controls in earlier experiments was 9 to 16 days, with a mean of 11.8 $\pm$ 0.15 days (S.D.), this slight prolongation in two subjects is probably significant. Chloramphenicol following chloroquine did not prevent relapses.

No evidence of prophylactic or curative activity was obtained with either penicillin or dihydrostreptomycin.

SUMARIO Y CONCLUSIONES

Aeromicina, cloramfenicol, penicilina, y dehidroestreptomicina se probaron como agentes profilácticos y curativos en pequeñas series muy bien controladas de infecciones inducidas de esporozoitos de la cepa Chesson de *P. vivax*. Los experimentos estuvieron principalmente orientados a probar la acción contra los parásitos en tejidos fijos y la cloroquina fué usada para eliminar los eritrocitos parasitados en las pruebas curativas.

Aeromicina en dosis de 4 gm. diarios durante 6 días después de la exposición produjo una demora significante en la aparición de los ataques agudos, confirmando resultados anteriores cuando 8 gm. diarios fueron suministrados (Cooper *et al.*, 1949). En consecuencia, la aeromicina 6 inhibe el desarrollo de los parásitos pre-eritrocíticos 6 por acción residual interfiere con la supervivencia y multiplicación de formas eritrocíticas jóvenes. La aeromicina no fué curativa y nó prolongó los intervalos de las recaídas más de lo que hace la cloroquina sóla.

Cloramfenicol en dosis de 4 gm. diarios durante 6 días también retardó ligeramente los ataques agudos, 18 a 22 días comparados con 11 a 14 días en los testigos. Como el período prepatente en 96 testigos consecutivos en experimentos anteriores fué de 9 a 16 días con un promedio de 11.8 ± 0,15 días (S. D.), esta ligera prolongación en dos sujetos es probablemente significante. Cloramfenicol en seguida de cloroquina no previene las recaídas.

No se tuvo evidencia de acción curativa o profiláctica ni de penicilina ni de dehidroestreptomicina.

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STUDIES IN HUMAN MALARIA

XXX. A Summary of 204 Sporozoite-induced Infections with the Chesson Strain of *Plasmodium vivax*

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The Chesson strain of *Plasmodium vivax*, isolated from a soldier who had recently returned to the United States from New Guinea (Ehrman *et al.*, 1945) has been widely used in chemotherapeutic research, and many features of experimental infections have been described (Gordon *et al.*, 1947, Whorton *et al.*, 1947, 1947a, 1947b, Craige *et al.*, 1947, Shannon *et al.*, 1948, Coatney and Cooper, 1948, Coatney *et al.*, 1949).

In the course of studies in two Federal penal institutions, we have observed 207 volunteers bitten by mosquitoes carrying this strain of parasite. Of these men, 204 developed overt malaria. Inasmuch as the volunteers were observed for 18 months after infection, a large body of data was accumulated which is illustrative of the long-term course of this strain of *vivax* malaria, particularly when successive attacks are interrupted with non-curative therapy. In addition, several experiments were carried out to elucidate certain effects of sporozoite dosage and of acquired immunity. It is our purpose to summarize in this paper the general findings in the entire group of infections.

MATERIALS AND METHODS

The methods used in these studies have already been described in detail (Coatney *et al.*, 1948) and consequently will be dealt with only briefly here. The subjects were white male volunteers in the Federal Penitentiary, Atlanta, Georgia or in the Federal Correctional Institution, Seagoville, Texas. Most subjects were bitten on a single exposure day by ten *Anopheles quadrimaculatus* mosquitoes infected with the Chesson strain of *Plasmodium vivax*. In certain experiments one, three or 30 infected mosquitoes per man were used, and in one experiment re-exposure was carried out at seven months and again at 13 months after the original bites. In all instances the mosquitoes were dissected after feeding and the density of sporozoites in the salivary glands was graded on a scale of 0 to 4+.

In some cases drugs were given protectively, i.e. for short periods before, and for short or long periods after exposure to infected mosquitoes. In the majority of subjects, however, primary attacks of malaria were allowed to appear without interference. They were interrupted with various drug regimens begun on the third to the fifth day of patency, and the subsequent relapses were carefully observed and recorded. In a few selected cases, attacks were left untreated.

Blood smears were made daily from the eighth through the 60th day after exposure,

with minor exceptions, and were then made two to three times weekly through the first year and at least once weekly until the end of 18 months. When patients developed patent parasitemia, fever, or symptoms suggesting malaria, they were hospitalized, a daily smear schedule was put into effect, and temperatures were taken every four hours.

Concentrations of drugs in plasma or whole blood were estimated; the details of these and other routine laboratory procedures are included in earlier papers of this series. Serial studies of complement fixation, using *Plasmodium knowlesi* antigen, were carried out in collaboration with the Department of Serology, Army Medical Research and Graduate School and these will be reported separately.

The experiments were carried out in an area where there was no natural transmission of malaria. Special surveys were made to rule out vectors in the immediate area, and patients with active malaria were kept on screened wards. No cases of malaria occurred in either institution other than those we experimentally induced.

GENERAL REVIEW OF MATERIAL

The 207 volunteers were exposed to mosquitoes at 17 separate feedings between 1 December 1944 and 17 September 1948. All but three men developed overt malaria; each of the latter had received protective medication.

Of the 207 volunteers, 153 were bitten by ten infected mosquitoes each; 96 of these received no protective drug before the first attack, while 57 did receive such medication. Fifteen men were bitten by one mosquito each, without protective medication; eight of these were exposed to reinfection. Twenty-one men were bitten by three mosquitoes each; all but one of these received protective drug. Eighteen volunteers were bitten by 30 mosquitoes each; all received protective drug.

There were 36 unavoidable separations from the group due to parole, transfer, or release, before the expiration of 18 months of observation. Of the original 207 men, 194 (93.7 per cent) were still under study at one year; 191 (92.3 per cent) at 15 months; 184 (88.9 per cent) at 17 months; and 171 (82.6 per cent) at 18 months.

PREPATENT AND INCUBATION PERIODS

Observations. In the 96 subjects bitten by ten infected mosquitoes and given no protective drug (table 1), malarial parasites were first detected in the blood smears nine to 16 days after exposure, the mean prepatent period being 11.77 days (S.D., 1.48 days, S.E. of the mean, 0.15 days). The median was 11 days. The first rectal temperatures of 101°F. were recorded ten to 17 days after mosquito bites, the mean being 12.16 days (S.D., 1.85 days, S.E. of the mean, 0.19 days).

Fifteen volunteers, bitten by one infected mosquito each, had prepatent periods ranging from 12 to 16 days, the mean being 13.93 days (S.D., 1.34, S.E. of the mean, 0.35); incubation periods to first temperature of 101°F. were 13 to 16 days, mean 14.00 (S.D., 1.01, S.E. of the mean, 0.26).

Comment. The prepatent and incubation periods in our subjects, bitten by ten mosquitoes each, were shorter than those reported by Whorton *et al.* (1947) for 134 volunteers infected with the Chesson strain at Stateville Penitentiary by a similar technique. They reported a mean prepatent period of 13.59 days (S.D., 1.45 days,

TABLE 1

Prepatent periods and incubation periods (to first rectal temperature of 101°F.) in 96 volunteers bitten by ten mosquitoes infected with the Chesson strain of Plasmodium vivax

DAYS	NUMBER OF VOLUNTEERS EXHIBITING INDICATED	
	Prepatent period	Incubation period
9	3	0
10	10	19
11	43	20
12	13	24
13	8	12
14	16	6
15	2	12
16	1	2
17	0	1
Total	96	96

FIG. 1. Frequency distribution, by week of onset relative to mosquito bites, of 802 acute attacks of Chesson strain *vivax* malaria, including both primary attacks and relapses.

S.E. of the mean, 0.13 days) and a mean interval to first rectal temperature of 100°F of 13.65 days (S.D., 1.73 days, S.E. of the mean, 0.15 days). It is believed that the differences are significant, inasmuch as both groups were followed similarly and with great care. Comparison of the data suggests that the infection densities in the mos-

quitoes used in our study averaged higher than those used by the Stateville investigators. Criteria for quantitating sporozoite densities in the two institutions were different, so that a direct comparison is not possible.

PATTERN OF RELAPSE ACTIVITY

Observations. Approximately 900 attacks of malaria, primary and relapse, were observed in the 204 men who had overt malaria. If the size of the inoculum and the type of therapy are disregarded, the frequency distribution by time of onset relative to exposure is as diagrammed in figure 1. This includes only 802 attacks, in men with single exposure dates, and omits all recurrences of patent activity after attacks that were untreated or interrupted with therapy known to be inadequate for the removal of erythrocytic parasites.

The graph also omits a few attacks that occurred later than 78 weeks (18 months) after mosquito bites, as there was no systematic follow-up beyond that time. There were four verified instances of later relapses, however, which began in the 80th, 88th 95th and 101st week after exposure, respectively.

Comment. The relapse pattern of Chesson strain *vivax* malaria has been contrasted previously with that of the St. Elizabeth strain (Coatney and Cooper, 1948). The latter strain was the subject of an earlier, detailed report (Coatney *et al.*, 1950). The frequency distribution of Chesson attacks shows clearly that there is no predictable period of long-term latency such as that which characterizes St. Elizabeth strain infections. The slight rise in frequency at 55 to 64 weeks, observable in figure 1, is the result of a group of delayed attacks following a year's suppressive medication.

This general pattern is the composite of many widely variant individual responses. Some volunteers had only one attack, others had as many as thirteen. Once the general strain characteristics have been demonstrated, it becomes of interest to define the factors which determine the actual sequence of events in an individual patient. Among such factors are variations in chemotherapy, the number and virulence of the infective sporozoites, and the limiting action of immunity.

SUCCESSIVE ATTACKS INTERRUPTED WITH NON-CURATIVE DRUGS

As will be shown in more detail later, when acute attacks of *vivax* malaria were allowed to go untreated, parasitemia persisted for variable periods, eventually becoming intermittently patent and then subpatent. Under such circumstances true relapses could not be distinguished from recrudescences resulting from surviving erythrocytic parasites. Our present interest attaches more to the relapse patterns which followed treatment of attacks with non-curative suppressive drugs, i.e. drugs which eradicate erythrocytic parasites, but do not affect the fixed-tissue parasites. In another category of activity, reserved for separate discussion, are such drugs as the 8-aminoquinolines, which eliminate or reduce the numbers of fixed-tissue parasites and thereby terminate infections or lower their relapse potential.

Observations on Volunteers Bitten by Ten Infected Mosquitoes. Presentation of data on non-curative drugs will be confined to quinine, chloroquine, SN 10,751 (amodiaquin or Camoquin), and chlorguanide, although several others were used. When malaria was allowed to start without the interference of a protective drug, and each attack

was interrupted by treatment with one of the foregoing drugs begun on the third to the fifth day of patency, erythrocytic parasites were promptly eradicated, but there were recurrent acute attacks until three to 16 months after exposure (table 2).

Several facts are evident from inspection of the data. (1) Relapses occurred more promptly after some drugs than after others. After quinine, parasitemia sometimes reappeared within one week; after chlorguanide in maximum dosage the lower limit

TABLE 2

The course of Chesson strain vivax *malaria in subjects bitten by ten infected mosquitoes, given no protective drug, and treated during each attack with the same non-curative regimen*

THERAPEUTIC REGIMEN USED AT EACH ATTACK			VOLUNTEER NUMBER	INOCULUM*	TOTAL NUMBER OF ATTACKS	SUCCESSIVE TREATMENT-TO-RELAPSE INTERVALS	ONSET OF LAST ATTACK (DAY AFTER BITES)
Drug	Total dose	Number of days					
	gm.					*days*	
Quinine	12.0	6	S-125	18+	8	9, 18, 17, 15, 17, 19, 36	200
Quinine	28.0	14	S-7	38+	11	7, 10, 10, 11, 14, 16, 16, 30, 103, 20	427
Quinine	28.0	14	S-11	36+	13	6, 7, 8, 8, 10, 13, 15, 16, 18, 29, 56, 48	455
Quinine	28.0	14	S-24	38+	11	6, 8, 11, 12, 13, 16, 34, 49, 50, 100	501
Quinine	28.0	14	S-41	40+	13	8, 8, 10, 10, 11, 15, 19, 24, 32, 36, 25, 44	465
Quinine	28.0	14	S-140	30+	6	35, 22, 46, 29, 45	278
Quinine	28.0	14	S-159	30+	4	8, 20, 397	489
Chlorguanide	14.0	14	S-108	26+	3	25, 30	100
Chlorguanide	14.0	14	S-109	39+	6	23, 25, 29, 28, 112	314
Chlorguanide	14.0	14	S-110	29+	6	25, 26, 28, 70, 157	403
Chlorguanide	14.0	14	S-111	29+	4	21, 25, 40	148
Chloroquine	1.5	4	S-69	25+	4	49, 58, 121	263
Chloroquine	1.5	4	S-70	25+	3	50, 98	172
Chloroquine	1.5	4	S-71	25+	3	42, 49	117
Chloroquine	6.0	14	S-113	26+	4	74, 79, 136	353
Chloroquine	6.0	14	S-114	40+	4	87, 86, 129	367
Chloroquine	6.0	14	S-115	29+	4	87, 80, 135	367
SN 10, 751	3.0	14	S-120	16+	5	81, 64, 111, 145	482
SN 10, 751	3.0	14	S-129	34+	6	46, 52, 56, 73, 143	466

* Expressed as the sum of the sporozoite densities (1+ to 4+) in the ten mosquitoes that bit each subject.

was three to four weeks; after chloroquine in standard dosage (1.5 gm. of base in four days), it was six to seven weeks; after chloroquine in large dosage (6 gm. of base in 14 days), it was ten to twelve weeks; after Camoquin, it was seven to ten weeks (See Coatney *et al.*, 1950a). (2) With few exceptions, the intervals from treatment to relapse in a given patient became longer as successive attacks were treated. (3) Within the range of infective inocula resulting from the bites of ten mosquitoes, the subjects who had widely-spaced attacks had fewer total attacks. Although there were great

differences in the time of onset of the last attack of malaria, this could not be correlated with the drug used.

Comment. The dosages of the various drugs used here were far above the amounts necessary to eradicate Chesson strain parasites from the blood stream (Wiselogle, 1946). The relapses are, therefore, considered as resulting from parasites that invaded the circulating blood from fixed-tissue sites. The intervals shown are in essential agreement with those obtained in other patients in whom the same regimens were used, but who, because of short periods of suppressive drug or shifts in therapy during the course of the infection, could not be included in this analysis.

The differences in treatment-to-relapse intervals observed after quinine, chloroquine or Camoquin treatment of early attacks correlate well with the relative persistence of the drugs, or active metabolites, in the human host (Wiselogle, 1946). The delayed appearance of parasites after chlorguanide, however, is not yet satisfactorily explained, as this drug is quickly eliminated from the body. If chlorguanide inhibits the pre-erythrocytic development of *P. vivax*, as suggested by the studies of Fairley (1947), then a shorter period of persistent action by the drug, or an active metabolite, would be necessary for delay of succeeding attacks than would be necessary if action were solely upon erythrocytic parasites.

The progressive increase in treatment-to-relapse intervals in the same patient could be due either to a gradual development of immunity to erythrocytic parasites, or to a gradual depletion of a reservoir of fixed-tissue forms. Experiments in which acute malaria was suppressed for six to twelve months (Lints *et al.*, 1950) suggest that partial immunity is probably the dominant factor. When quinine was used to interrupt the acute attacks that appeared after a year's suppression, treatment-to-relapse intervals were at first almost as short as those in early primary attacks in controls.

While immunity resulting from repeated experience with erythrocytic parasitemia appears to have an effect upon the intervals between attacks, in infections of the severity observed in this series it was not the most important determinant of the number of attacks or of the duration of the period during which acute attacks occurred in a patient. If it had been the dominant factor, one would have expected that the patient with widely-spaced attacks due to therapy with a persistent drug would have had as many attacks as the patient with closely-spaced attacks, but that they would have been spread over a longer period of time. This was not the case, however, which points to some factor other than immunity to erythrocytic parasites which affects the total duration of an infection. Possibly the important factor is the size of the infective inoculum (Boyd, 1940).

Observations in Volunteers Infected by Bites of Single Mosquitoes. In an attempt to obtain infections displaying fewer attacks and a shorter total duration than those resulting from the bites of ten infected mosquitoes, a group of 15 volunteers was studied in whom the original infective inoculum was supplied by one infected mosquito biting each. Each attack of malaria was interrupted by a single 0.3 gm. dose of chloroquine.

The resultant pattern of malarial attacks was quite varied (table 3 and figure 2). At seven months (day 214), when the volunteers had displayed from one to six attacks each, eight men were rebitten, again by a single infected mosquito per man. The group for rebiting and the controls were chosen in such a manner that the num-

ber of previous attacks in each group was approximately equal, e.g. of the two men who had had one attack each, one was rebitten, of the five who had had three attacks, three were rebitten, etc. Four new controls (S-233, S-234, S-235 and S-236) were included at the second feeding and were bitten by single mosquitoes. The mosquitoes

TABLE 3

Sequence of attacks in volunteers in whom Chesson strain vivax *malaria was initiated by the bite of one infected mosquito per man*

Selected volunteers were rebitten as indicated. Each attack of malaria was interrupted by 0.3 gm. of chloroquine (base). Volunteers in each group are arranged in order of the number of attacks that had been experienced at time of first refeeding.

VOLUNTEER NUMBER	PERIOD NO. 1 FIRST EXPOSURE TO REBITE ON DAY 214				PERIOD NO. 2 DAY 214 TO DAY OF SECOND RE-EXPOSURE ON DAY 393			PERIOD NO. 3 DAY 393 TO END OF TEST ON DAY 547			GRAND TOTAL
	Inoculum*	Days to first attack	Number of attacks	Days of patent parasitemia	Inoculum	Days to first attack	Number of attacks	Inoculum	Days to first attack	Number of attacks	Number of attacks
S-205	+++	14	1	7	+++	—	0	29+	12	6	7
S-206	++	16	2	11	+++	18	1†	—	—	—	—
S-197	++++	13	3	19	++++	14	3	26+	18	2	8
S-200	++	14	3	15	++++	12	4	33+	34	3	10
S-207	++	14	3	17	+++	16	3	29+	10	1	7
S-196	++++	14	5	27	+++	17	3	28+	—	0	8
S-199	++++	14	5	32	++	23	1	33+	18	1	6
S-202	++++	12	6	34	++++	12	2	29+	—	0	8
S-209	++	16	1	4	—	—	0	—	—	0	1
S-198	+++	16	3	20	—	—	0	—	118	1	4
S-208	++++	14	3	16	—	—	0	—	—	0	3
S-203	++	15	4	20	—	140	1	—	—	0	5
S-201	++	12	5	25	—	15	2	—	104	1	8
S-204	++++	13	5	30	—	3	2	—	98	2	9
S-210	++++	12	6	32	—	42	2	—	62	1	9
S-233					+++	17	1	23+	12	4	5
S-234					+++	—	—	0	—	—	0
S-235					++	—	—	0	—	—	0
S-236					++++	17	1	2+	14	2‡	‡

* Expressed as the sum of the sporozoite densities (1+ to 4+) in the mosquito or mosquitoes that bit each subject.

† Released from custody on day 365, 151 days after second feeding, with adequate followup through day 547.

‡ Released from custody 90 days after second feeding.

used were weakly infected and only two of the four controls developed malaria, 17 days after bites. Nevertheless, seven of the eight men who were rebitten had patent parasitemia beginning 12 to 23 days after their second exposures. In the unrebitten controls, only one relapse occurred during the corresponding period, although some of the men later displayed additional relapses resulting from their initial exposures.

Again, on 30 August 1949 (393 days after the original exposure) the same individuals were again rebitten, this time by ten heavily infected mosquitoes per man. Although in five of seven instances an attack of malaria began ten to 34 days after the bites, in all subjects except S-205, these were low-grade, and there was evidence of considerable tolerance and immunity. Subjects S-233 and S-236, who had been infected as controls at the second feeding, were included in the third feedings, and each responded with a series of acute attacks which continued for several months.

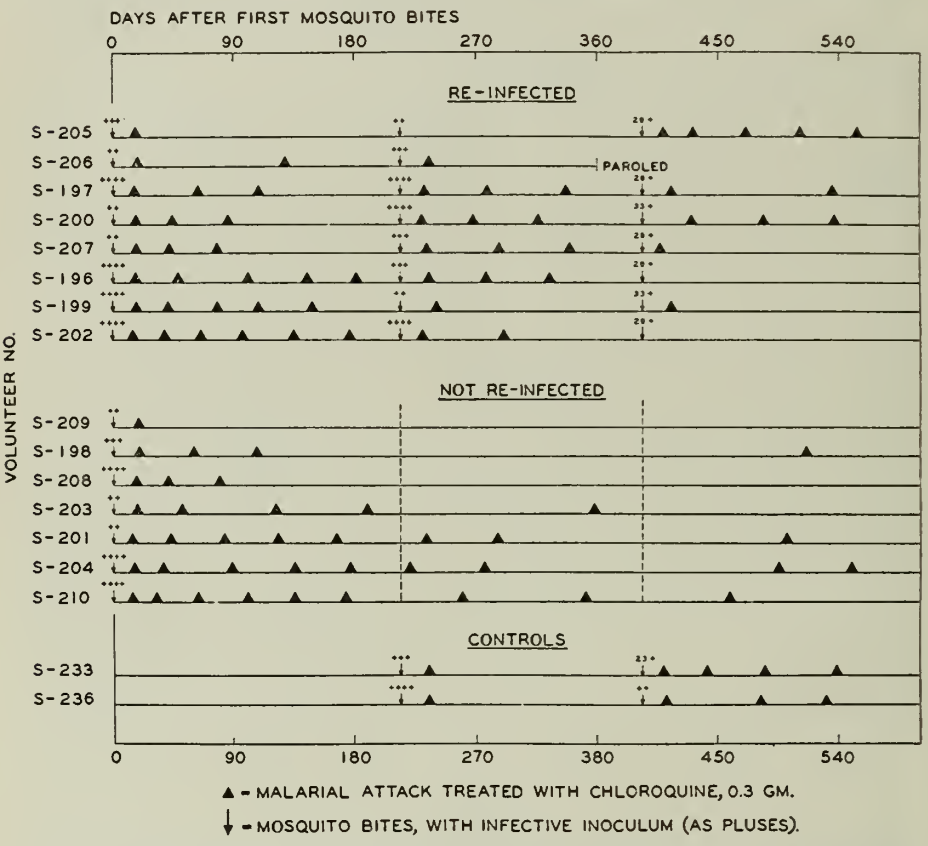

FIG. 2. Pattern of attacks of Chesson strain *vivax* malaria in subjects bitten by one infected mosquito each. (Presentation of attacks is diagrammatic). Selected individuals were reinfected as indicated.

Comment. When volunteers were infected by single mosquito bites, we were impressed by the great variability in their responses. Whereas some men had only one, two or three attacks, others continued to have relapses for nearly 18 months. We are more concerned at the moment with those who had only one to three attacks, because such brief activity rarely occurred in men bitten by ten heavily infected mosquitoes.

Yount and Coggeshall (1949) demonstrated that after a single attack of Chesson strain *vivax* malaria, cured with pentaquine-quinine, there was no appreciable homologous strain immunity. After four to seven attacks, followed by chemotherapeutic

cure, homologous strain immunity was present, but was inadequate to prevent an abortive attack following sporozoite inoculation.

The successful reinfection of subjects S-197, S-200, and S-207, contrasted with the pattern in S-198 and S-208, is in agreement with the findings of Yount and Coggeshall (1949). Although the infections in their cases had been stopped by chemotherapy, ours had presumably come to an end by natural means, but without immunity sufficient to prevent reinfection, even though only three to four months had elapsed since the last attacks. The failure of S-205 to become reinfected is not regarded as significant, inasmuch as it was possible to infect only two of four controls by mosquitoes from the same lot; S-205 was completely susceptible to reinfection when ten mosquitoes were used on day 393. On the latter date, other rebitten volunteers (S-197, S-200, S-207, and S-199), who by this time had experienced six to seven attacks apiece, responded with modified attacks, whereas volunteers S-196 and S-202, who had had eight attacks each, had no further malarial attacks.

Relapse activity in S-233 and S-236, who had one attack each after being bitten by one mosquito on 4 March, 1949, and who were rebitten 179 days later, is in agreement with the foregoing. After the second feeding S-233 responded with an acute febrile attack within 12 days and had a series of relapses, so that four attacks had occurred during an observation period comparable to that of other members of the experiment. S-236 was rebitten by only one infected mosquito and displayed an acute febrile attack 14 days later, followed by two more relapses at intervals of 66 and 47 days.

Although the data are too few for sweeping conclusions, they strongly suggest that a large proportion of *vivax* infections resulting from small numbers of sporozoites will display short courses and few relapses and that they may subside under non-curative therapy without the development of homologous strain immunity. Volunteers who display only one to three attacks of malaria following one mosquito bite are not necessarily those with a high degree of immune response, but perhaps those who were fortunate enough to receive fewer sporozoites. Their immunity three to four months after the last attack appears no greater than would have been expected had they been cured by pentaquine and quinine after their first, second, or third attacks. After six or seven attacks a patient's acquaintance with the erythrocytic parasites is sufficient to produce definite tolerance and modification of the relapse pattern when challenged by bites from ten infected mosquitoes.

PROTECTIVE USE OF NON-CURATIVE DRUGS

Observations. Suppressive drugs such as quinacrine, chloroquine, chlorguanide and SN 10,751 (amodiaquin or Camoquin) completely suppressed the patent parasitemia and clinical manifestations of Chesson strain malaria, induced by ten mosquito bites, both while drugs were being administered and for varying lengths of time thereafter. Also, the latent intervals from the end of suppression until the appearance of delayed primary attacks were of the same relative order of magnitude as that of the treatment-to-relapse intervals when the same drugs were given therapeutically (Coatney *et al.*, 1950b, Lints *et al.*, 1950, Coatney *et al.*, 1950).

That the number of infective sporozoites has some effect upon the dosage of drug

required for suppression is illustrated by an experiment summarized in table 4. In parallel groups of volunteers, infected by mosquitoes from the same lot on the same day, men bitten by three infected mosquitoes each were completely protected from

TABLE 4

Suppression of Chesson strain vivax *malaria, induced by the bites of 30 or 3 infected mosquitoes, with various regimens of chloroquine, continued for 6 weeks after exposure*

Each post-suppression attack interrupted with 0.3 gm. of chloroquine.

VOLUNTEER NUMBER	INOCULUM		PROTECTIVE CHLOROQUINE GM. PER WEEK DAYS −3 THRU 39	EFFECTIVENESS OF SUPPRESSION	DAYS FROM END OF SUPPRESSION TO FIRST PARASITEMIA	NUMBER OF ATTACKS	ONSET OF LAST PATENT PARASITEMIA	DAYS OF OBSERVATION
	Number of mosquitoes	Sum of pluses*						
S-160	30	116+	0.3	Temp. 100.6°F. day 11	42	6	319	547
S-161	30	114+	0.3	Satisfactory	39	8	443	547
S-162	30	111+	0.3	Positive smear day 10, Temp. 101.8 day 11	41	9	480	547
S-163	30	119+	0.2	Satisfactory	41	5	282	547
S-164	30	110+	0.2	Satisfactory	41	5	331	547
S-165	30	114+	0.2	Satisfactory	39	7	501	547
S-166	30	113+	0.1	Positive smear day 10, Temp. 101.8 day 10; 102 day 11	20	6	247	547
S-167	30	113+	0.1	Positive smears days 10, 12; Temp. 104.6 day 11, 103.2 day 12	27	11	500	547
S-168	30	110+	0.1	Positive smear day 11	28	5	269	547
S-169	3	9+	0.3	Satisfactory	98	1	137	284†
S-170	3	10+	0.3	Satisfactory	63	4	470	547
S-171	3	12+	0.3	Satisfactory	61	1	100	547
S-172	3	11+	0.2	Satisfactory	53	7	437	547
S-173	3	9+	0.2	Temp. 101.8 day 16, 101.8 day 17, 101.2 day 19	43	3	541	547
S-174	3	11+	0.2	Satisfactory	43	4	428	547
S-175	3	9+	0.1	Satisfactory	28	2	168	547
S-176	3	10+	0.1	Satisfactory	27	8	483	547
S-177	3	11+	0.1	Satisfactory	33	1	72	547

* Expressed as the sum of the sporozoite densities (1+ to 4+) in the mosquitoes that bit each subject.

† Released on parole.

patent parasitemia by 0.3, 0.2, or 0.1 gm. of chloroquine (base) once weekly, begun on the third day before exposure. Those bitten by 30 infected mosquitoes were protected by 0.3 or 0.2 gm. of chloroquine, but not by 0.1 gm. once weekly. The last

group displayed fever and transient patent parasitemia on days 10, 11 and 12. The breakthroughs were abortive, in that they were alleviated by the regularly-scheduled weekly dosage of chloroquine (on day 11), and no further evidence of malaria occurred until suppression was discontinued six weeks after mosquito bites.

The successive attacks of malaria that occurred in these 18 patients after the end of six weeks of suppression were all interrupted with chloroquine, in single doses of 0.3 gm. of base. This is just above the minimum necessary to clear erythrocytic parasites, and recrudescences due to surviving erythrocytic parasites cannot be completely ruled out in all cases. Nevertheless, the average number of attacks in the men bitten by 30 mosquitoes was greater than that in the men bitten by three mosquitoes. The former displayed a total of 62 attacks, the mean number per man being 6.9 (S.D., 1.72, S.E. of the mean, 0.61) whereas the latter had 31 attacks with a mean of 3.44 (S.D., 2.42, S.E. of the mean, 0.85).

In a similar experiment which has already been reported (Cooper *et al.*, 1950) parallel groups of volunteers were exposed to 30 or three mosquitoes per man, but the suppressive drug used was chlorguanide, in dosage of 0.2, 0.1 or 0.05 gm. of hydrochloride per week. There were no breakthroughs, but malaria appeared in all but one man (from the lightly-infected group) after cessation of medication, six to 12 months after bites.

Comment. In infections resulting from 10 infective mosquito bites, chloroquine, chlorguanide or Camoquin provided complete protection when given once weekly. Infections outlasted six months' or one year's suppression with chloroquine or chlorguanide, the period of residual protection after stopping medication being longer with the former drug. When heavier inocula were used (e.g. 30 mosquito bites) and a suboptimal dosage of chloroquine was given (0.1 gm. per week), there was a tendency to breakthrough at the end of the normal prepatent period, ten to 11 days after bites, but there were no subsequent breakthroughs. It was found that in all but one case infections caused by three mosquito bites outlasted one year's suppression with chlorguanide. It must be recognized, however, that when heavily-infected mosquitoes are used, even three bites supply a considerable challenge. In view of the fact that infections resulting from small inocula often yield only one or two acute attacks over a brief period of time, one can predict with confidence that in practice some infections would be permanently eliminated by suppression continued for several months after the last possible exposure, except where all subjects had been heavily exposed.

USE OF CURATIVE DRUGS

Of the drugs used in these experiments, only certain 8-aminoquinoline derivatives (pentaquine, isopentaquine, and analogues) consistently reduced the relapse incidence and total duration of infections when compared with controls treated with "non-curative" drugs. The curative power of 8-aminoquinolines, particularly when given in combination with quinine, has been adequately documented by many workers. We were impressed, however, by the variability of response in different groups of volunteers. Although there was a partial correlation between difficulty of cure and the number of sporozoites observed upon dissection of the infective mosquitoes,

neither numbers of sporozoites nor prepatent periods of primary attacks were infallible guides for predicting relapse rates (Coatney *et al.*, 1950b).

UNTREATED ATTACKS

Observations. Chemotherapy was not entirely withheld in any subject. In 23 men, however, attacks other than the primary were left untreated. A representative group of such untreated attacks is diagrammed in figure 3. It can be observed that in the four patients in whom first relapses were untreated, parasitemia was prolonged for several months, 104 to 135 days of patent parasitemia occurring before the infections

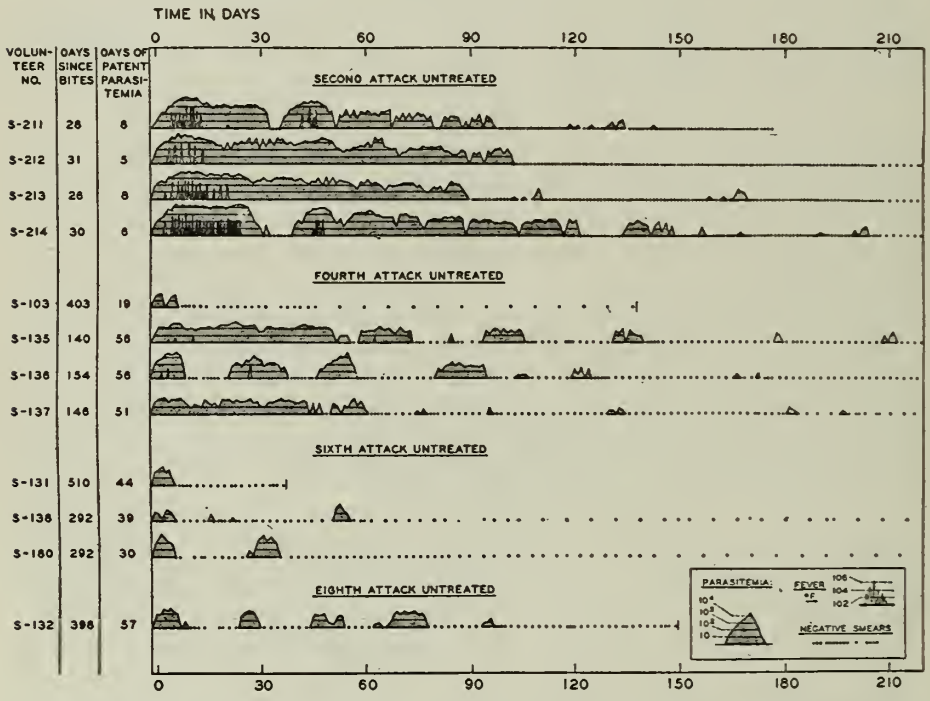

FIG. 3. Course of parasitemia and fever in Chesson strain *vivax* malaria left untreated after varying periods of prior activity.

became and remained subpatent. Peak parasite counts in these men occurred on the sixth to the eleventh day of the first uninterrupted parasitemic rise, reaching concentrations of 11,000 to 31,500 per cu. mm. of blood. There were subsequent cyclic rises and falls, with progressively declining peak parasite counts. The total febrile periods in these men ranged from 14 to 32 days.

In subjects who had had three attacks before one was left untreated, there was in most instances a period of sustained patent parasitemia, but parasite concentrations were not as high and the febrile periods were short or absent.

In subjects who had had five or seven attacks before being left untreated, there was usually prompt spontaneous clearance of parasitemia within a few days of onset of

an attack without evidence of fever. There were a variable number of subsequent mild recrudescences before the infection became permanently latent or spontaneously cured.

DISCUSSION

After therapy sufficient to eradicate erythrocytic parasites, a relapse of sporozoite-induced *vivax* malaria theoretically depends upon (1) the continued presence of fixed-tissue parasites; (2) the maturation of these parasites to the stage capable of invading the erythrocytes, a process which in some strains at least is thought to be periodic rather than continuous; (3) the absence of immunity to erythrocytic parasites which would prevent their multiplication to patent levels; (4) the absence of suppressive concentrations of antimalarial drug in blood or tissues.

The observed facts in experimental *vivax* malaria are in agreement with the foregoing working hypothesis. Our major zone of ignorance relates to the behavior of the fixed-tissue parasites. Are they continually reproducing or are they periodically dormant? If the discharge of parasites capable of invading erythrocytes is periodic, what governs the periodicity in strains such as the St. Elizabeth and other temperate zone strains which show such a high incidence of relapses nine to ten months after the original inoculation? Are the forms which persist and cause relapses lineal descendants of the early pre-erythrocytic forms which have been described by Shortt and Garnham (1948), or do they stem back to sporozoites by some collateral line? These and other questions are still unanswered.

We propose to limit the following discussion to conclusions, observations and speculations on the course of events in Chesson strain *vivax* malaria, when initiated by light or heavy sporozoite inocula, under various types of management.

When a large number of sporozoites is introduced, there is initiated a period lasting 12 to 18 months or more, during which there is a fairly regular invasion of the erythrocytes from the fixed tissues. That there is invasion by an unusually large number of parasites at the end of the initial eight to ten day period following infective bites is evidenced by the tendency to early breakthrough in the face of borderline doses of chloroquine. That reinvasions in heavy infections are almost continual is evidenced by the relapses that occur in such cases almost as soon as residual drug from therapy drops to non-suppressive concentrations. Developing tolerance to the parasite is evidenced by the higher parasite counts that are necessary to produce fever in successive attacks (cf. Blackburn, 1948). Immunity is at first manifested by increasing intervals between attacks, despite identical therapy, and later by the spontaneous clearance of parasitemia even though treatment is withheld. Suppression of heavy infections for as long as six or 12 months is followed by overt malaria soon after the last dose of drug. Massive inoculation of sporozoites apparently creates a very large reservoir of fixed-tissue parasites, and in such infections tolerance and immunity to the erythrocytic parasites is of importance in altering the severity, spacing, and number of acute malarial attacks that ensue. Suppression of such malaria, if it is to reduce the incidence of the disease when discontinued, probably must continue for years, rather than months, after the last heavy exposure.

In infections where the inoculum is very small, as might occur with the bite of one

lightly infected mosquito, certain individuals would be expected to have no malaria, others only one, two or three acute attacks, even if non-curative drugs were used. In weak infections, therefore, immunity to erythrocytic parasites is of less importance as a determinant of the number of attacks or the total duration of infection. And, although we still do not have clearcut evidence to prove it, it is in accord with the short duration of some of these infections to predict that prolonged suppression would outlast the fixed-tissue stage of the infections in a certain proportion of individuals and thereby permanently prevent the appearance of active malaria.

No mention has been made of the possibility that immunity to exoerythrocytic parasites may be acquired. No inferences as to the existence or relative importance of such an immune response can be drawn from our studies. It would be of interest, for example, to know whether or not individuals repeatedly exposed to the same strain of *P. vivax*, in the face of suppressive medication sufficient to protect against erythrocytic parasitemia, would develop immunity to that strain.

The results in our controlled studies serve to re-emphasize the variability of *vivax* malaria. When the strain of parasite and the drug regimen are kept constant, and the disease is studied only in healthy, white males, differences in the number and virulence of sporozoites and in the responses of individual patients are variables which produce variant relapse sequences. When many strains are superimposed, in individuals of differing ages and states of nutrition, who are subject to repeated reinfections as occurs in malaria in its natural habitat, there is little wonder that the pattern of relapsing vivax malaria can appear to be hopelessly complex.

SUMMARY AND CONCLUSIONS

The course of 204 sporozoite-induced infections with the Chesson strain of *Plasmodium vivax* has been reviewed and the results may be summarized as follows:

1. When ten mosquitoes were used to transmit the infection, the prepatent and incubation periods averaged 11.8 and 12.1 days respectively, as compared to 14.0 days for each period when the infection was induced by one mosquito bite.

2. No long-term latent period has been demonstrated for this strain.

3. When acute attacks were allowed to go untreated, the parasitemia persisted for variable periods, eventually becoming intermittently patent and finally disappeared altogether.

4. Infections induced by the bites of 10 infected mosquitoes, a relatively heavy inoculum, showed recurrent acute attacks for three to 16 months, when each attack was treated by a non-curative drug. The intervals between treatment to relapse usually became progressively longer as the infection advanced.

5. Infections induced by one infected mosquito exhibited shorter courses and fewer relapses.

6. Suppressive drugs such as quinacrine, chloroquine, chlorguanide and SN 10,751 completely suppressed the parasites and symptoms of infections induced by the bites of ten infected mosquitoes, during the period of drug administration and for varying lengths of time thereafter. The evidence shows that a greater suppressive dose is needed for persons receiving a large inoculum of malaria sporozoites than for those receiving a small inoculum.

7. Of the drugs used in these experiments only certain 8-aminoquinoline derivatives (pentaquine, isopentaquine and analogues) consistently reduced the relapse incidence and total duration of infections. This was not true of controls treated with "non-curative" drugs such as quinine, chloroquine, SN 10,751 (Camoquin) and chlorguanide.

8. In patients where the first relapses were not treated the parasitemia was patent for several months, showing cyclic rises and falls with progressively declining peak parasite densities. In patients where later relapses were not treated, similar results were obtained except that the duration of the parasitemias was shorter and lower densities prevailed.

9. The evidence indicates that the Chesson strain of *Plasmodium vivax*, when induced by mosquito bites, can exist in the body for as long as 18 months, during which time there is a fairly periodic invasion of the erythrocytes by parasites arising from the fixed tissue. The duration of the infection and the number of relapses appear to be related directly to the size of the inoculum; consequently, the length of time that drugs must be given to suppress the disease is also related to the size of the inoculum.

10. This study has re-emphasized the variability of the behavior of vivax malaria under conditions where many factors were controlled. Under uncontrolled natural conditions, the pattern of relapsing vivax malaria certainly can be more complex.

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In the several years required for these studies many professional and technical personnel took part in the actual day-to-day responsibilities. The medical staff members included Drs. D. S. Ruhe, J. J. Griffitts, M. Landowne, J. K. McBane, T. L. Perrin, W. B. Culwell, Weldon C. White, H. A. Lints, and C. A. Imboden, Jr. Mrs. Marjorie Bee, Miss Virginia D. Hines, and Mr. H. L. Bryant were at various times in charge of nursing service; Drs. D. E. Eyles, G. M. Jeffery, and J. M. Ellis were at various times responsible for supplying infected mosquitoes; Dr. Edward S. Josephson, Mr. J. J. Burns, Mrs. Alice M. R. Thompson, Miss Marie A. Gilson, Miss Elizabeth Guinn, Mrs. Julia R. Wess, Mrs. Virginia Duff-White, and Mr. F. J. Fearney carried the burden of chemical and parasitological examinations. To all these people we extend our grateful acknowledgment.

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EPIDEMIOLOGICAL APPRAISAL OF REPORTED MALARIA IN THE UNITED STATES FROM 1947 THROUGH 1949

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This paper represents the results of a qualitative and quantitative study of reported malaria by the Public Health Service and the health departments of five Southeastern States.

The primary purpose of the epidemiological appraisal program was to ascertain the relative accuracy of the morbidity and mortality reported as malaria. It was intended to use this epidemiological evidence to guide selection of areas in which both antivector and antiparasite control measures should be concentrated and to measure the success of efforts to eradicate residual foci of infection or transmission.

Secondarily, epidemiological appraisal was intended to discourage spurious reporting and to improve reporting by searching out and confirming unreported cases.

Thirdly, the evaluation of malaria biometry was chosen as an interpretative example to determine the relative accuracy of our morbidity and mortality reporting systems as they relate to communicable disease control in general.

Cases of malaria usually are reported weekly by the diagnosing physician to the local health department, which in turn tabulates and forwards these records to the state health department. After being recompiled, the totals are telegraphed to the Public Health Service in Washington. In order to appraise the validity of reporting, it was necessary to obtain the basic epidemiological data from the physician and patient. Since the legal responsibilities of the reporting system are invested in the states, this appraisal program was administered by the state health departments, utilizing principally Communicable Disease Center personnel detailed to the states.

Epidemiological personnel were assigned to the states reporting the most malaria to assist state epidemiologists in carrying out this program. In the summer of 1947 a medical officer was assigned to each of the states of Arkansas, Alabama, Georgia, and Mississippi, and a nurse each to South Carolina and Mississippi.

During the latter half of 1947, the states investigated the largest groups of reports which characteristically stemmed from a small number of physicians in a few counties. Fevers of undetermined origin and other ill-defined clinical entities were found ascribed to malaria. The diagnoses of many cases were based on grounds that the patients merely felt bad. A therapeutic response was considered diagnostic by many physicians. The multi-disease card reporting system which did not identify or locate the patients was considered responsible for the vast majority of spurious reports. In Mississippi, the institution of a system giving the disease, patient's name and address eliminated the greater part of the statistics not founded on sound clinical or laboratory criteria.

In both 1947 and 1948, blood surveys were carried out in those counties reporting large numbers of cases. No positive smears were found. Interest was focused on ac-

curate diagnosis and reporting by the showing of movies, the conducting of seminars in parasitology; lectures; and the use of the radio and press. Not only were groups of officially reported cases investigated, but also laboratory records, physicians' references, and rumors suggesting malaria. When confirmed, these case records were incorporated into the official statistics.

In January 1948, the appraisal of individual reports was begun in three states. This was done objectively by utilizing a special appraisal form produced with the cooperation of all participating states. On this form were entered only basic epidemiological data which led to the morbidity or mortality report. Where possible, the reviewer traced the report through the county health department to the diagnosing physician and ultimately to the patient. Attempts were made to obtain the name, address, age, sex, and race of the patients. The case was dated both by report and onset. The source and date of the original attack were noted where known. The reporting physician was interviewed or his records reviewed to determine the method of diagnosis, whether by history, clinical impression, or laboratory means. Whereever available, the blood smears made by the diagnostician were reviewed by the appraiser or referred to the state health department or Public Health Service malaria laboratories for confirmation. With the consent of the physician, a blood specimen was obtained from the patient.

The quality of the technical standards of the laboratory examination was appraised as *acceptable*, *erroneous*, or *undetermined*. In a small number of instances, the patient was reexamined clinically with the diagnostician or with his concurrence. Where a differential diagnosis was indicated, the physician was encouraged to use the facilities of the state or other specialized public health laboratories. Thus the appraisals were made by history, clinical examination, and laboratory means.

During 1948 the appraised cases were classified as *positive*, *doubtful*, or *improbable*. Under the *positive* category were included all cases with laboratory confirmation by technical standards acceptable to the state health department, as well as those presumptively positive cases with consistent clinical histories, clinical findings, and therapeutic response. Under the *improbable* category were included cases which lacked laboratory confirmation, which did not present consistent histories and clinical findings, or which suggested some disease other than malaria. Under the *doubtful* category were included all cases the appraiser could assign to neither the *positive* nor *improbable* categories, usually because of incomplete data.

Although the aforedescribed classification removed the *improbable* and *doubtful* reports from too serious epidemiological consideration, the *positive* category was too inclusive. During 1949 this category was separated into a *positive* category to include only reports confirmed by laboratory standards acceptable to the state health department, and a *presumptive* category to include those cases with clinical diagnoses considered valid by the appraiser.

The above methods were used to investigate the individual reported cases except where medical relations, incomplete data, or other factors contraindicated or modified the procedures. Where these appraisal procedures revealed the need, special blood surveys were conducted to sample parasitemia in the population. In September 1948, the emphasis of appraisal was shifted in South Carolina from large groups

of reports to individual cases. Not until September 1949 did Arkansas initiate individual case appraisals. Prior to that time, they supplemented the laboratory facilities of the reporting physicians, held parasitologic seminars for physicians and their technicians, and performed blood surveys with mobile laboratories to evaluate the larger groups of reports. No positive films have been found by the Arkansas State Hygienic Laboratory among at least 2,000 slides during the course of this study.

RESULTS

The results of the program during the first few months in the fall of 1947 were hardly demonstrable except in the counties accounting for the majority of cases. In Alabama, 754 cases were reported from one county and 408 from another. These cases constituted over two-thirds of those reported for the entire state. None of

TABLE 1

Appraisals of reported malaria January 1948 to September 1949, inclusive

STATE	REPORTS	APPRAISED	APPRAISAL OF DIAGNOSIS				METHOD OF DIAGNOSIS			EVALUATION OF LABORATORY RESULTS			PROBABLE SOURCE		
			Positive*	Presumptive	Doubtful	Improbable	Clinical and laboratory	Laboratory	Clinical	Acceptable	Erroneous	Undetermined	Within U.S.	Outside U.S.	Undetermined
Ala.	429	196	42	105	35	14	114	0	82	51	2	61	146	49	1
Ga.	158	153	65	10	29	49	47	78	28	64	24	37	81	61	11
Miss.	170	145	55	24	28	38	84	2	59	59	8	19	111	34	0
S. C.	4058	548	18	86	256	188	55	2	491	28	0	29	524	6	18
Total	4815	1042	180	225	348	289	300	82	660	202	34	146	862	150	30

* Confirmed by laboratories aproved by State Health Department.

these was confirmed by examining the smears taken by the physicians. Supplemental blood surveys were negative. Therefore, the state decided to withdraw from its official reports all cases except for an estimated 40 for the first county and 20 for the other. In Arkansas, 368 cases were reported from a single county. These could not be confirmed but as a result of investigation, reports practically ceased.

Table 1 shows the results of appraising 1,042 cases individually from the 4,815 reports from the states of Alabama, Georgia, Mississippi, and South Carolina, from which individual appraisal forms were received up to October 1, 1949. The appraised cases represent 22 per cent of those officially reported. Of these 1,042 cases appraised, 180 were in the *positive* category, which for the purposes of this summary included only diagnoses confirmed by blood films examined under laboratory standards acceptable to the state health departments.

Of the 180 positive cases, 143 were due to *Plasmodium vivax*, 29 to undetermined species, 5 to *P. malariae*, and 3 to *P. falciparum*. Of those same 180 confirmed cases,

115 were believed to have originated outside the United States and only 59 from within this country (origin of 6 undetermined).

DISCUSSION

Under the conditions of diminishing endemicity of malaria in this country from 1947 through 1949, the epidemiological follow-up of reported cases has reduced spurious reporting more than it increased the reporting of undetected or unreported malaria. Physicians have been discouraged from reporting their clinical diagnoses of malaria. Nevertheless, the epidemiological appraisal program has reduced morbidity reporting to a level reflecting more nearly the actual incidence of malaria.

CONCLUSIONS

(1) Two years' epidemiological studies of malaria have further supported the concept that where reported malaria is investigated, it tends to disappear.

(2) Most of the cases reported to the state health departments lacked consistent clinical history or laboratory proof of infection. Few of these could be confirmed by case follow-up.

(3) During 21 months of investigations in four states, only 59 reports were confirmed by laboratory standards acceptable to the state health departments and were believed to have had their origin within the United States.

CONCLUSIONES

(1) Dos años de estudios epidemiológicos sobre malaria han respaldado el concepto de que dondequiera que la malaria denunciada se investiga ésta tiende a desaparecer.

(2) La mayoría de los casos denunciados a los Departamentos Estadales de Salud Pública carecen de historia clínica completa o prueba de laboratorio. Pocos de estos pueden comprobarse al verificarlos.

(3) Durante 21 meses de investigaciones en cuatro estados, solamente se comprobaron 59 casos con origen probable dentro de los Estados Unidos mediante técnicas de laboratorio aceptables por los Departamentos Estadales de Salud Pública.

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